Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 72 NOVEMBER, 1949 No. 2

SECTION MEETINGS

ILLINOIS	
Searle Laboratories	October 18, 1949
MINNESOTA	
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PACIFIC COAST	
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University of Buffalo	October 22, 1949

Influence of Hyaluronidase and Steroids on Permeability of Synovial Membrane. (17406)

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It has previously been reported[1] that hyaluronidase reversibly accelerated osmosis and abolished the semipermeable nature of membranes prepared from the excised urinary bladder of rabbits. Skeletal and cardiac muscle membranes were not affected. Adrenal cortical extract and estrone diminished permeability of bladder membranes and antagonized the effect of hyaluronidase. Desoxycorticosterone acetate enhanced the action of hyaluronidase.

The effect of hyaluronidase and steroids on membranes *in vivo* has not been studied heretofore. For this purpose we selected the synovial membrane of rabbits and humans. The synovial membrane and synovial fluid contain hyaluronic acid and are altered in arthritis.[2] The alteration has been attributed to a specific effect on the hyaluronic acid by hyaluronidases[2] and a structural change in the joint brought about by desoxycorticosterone intoxication as a disease of adaptation.[3] Arthritis has been treated successfully by the administration of Compound E or of adrenocorticotrophic hormone, which presumably releases this compound from the adrenal cortex.[4] It was therefore of interest to determine whether hyaluronidase and desoxycorticosterone weaken the normal synovial barrier by increasing its permeability and whether other adrenal steroids, either administered by injection or released endogenously by the alarm reaction, would strengthen the barrier and antagonize the effects of hyaluronidase.

The experiments on rabbits are reported in this paper. The data obtained on humans under the influence of various drugs and diseases will be reported in a subsequent paper. by Fitch, D.R., Corn, O., Jallo, Jr., S. J., and Seifter, J.

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[1] Seifter, J., Baeder, D. H., and Dervinis, A., PROC. SOC. EXP. BIOL. AND MED., 1949, **72**, 136.

[2] Meyer, K., *Physiol. Rev.*, 1947, **27**, 335.

[3] Selye, H., *J. Clin. Endocrin.*, 1946, **6**, 117.

[4] Hench, P. S., Kendall, E. C., Slocumb, C. H., and Polley, H. F., *Proc. Staff Meet., Mayo Clinic*, 1949, **24**, 181.

Material and methods. Sixteen male white rabbits weighing between 3.5 and 5.0 kg were prepared for this study by a modification of the method reported by Curtis and Brunschwig for studying the human knee joint.[5] Each animal was anesthetized with 40 mg sodium pentobarbital per kg intraperitoneally, then placed on its dorsal surface and the inner aspect of each right hind leg was shaved at the talocrural articulation. A soft rubber catheter was inserted through the urethra into the bladder and retained there for the duration of the experiment. A sample of urine was removed immediately to show that no dye was present in the urine at the beginning of the experiment. Then by inserting a 26 gauge one-inch needle from the inner aspect of the ankle, 1.25 mg phenolsulphonphthalein (PSP) in 0.25 ml of 0.85% NaCl solution was injected into the cavity of the talocrural articulation. After insertion of the needle the syringe was aspirated to insure the presence of the needle in the joint cavity without having caused trauma and to check against the rupture of any small blood vessels. The joint was kept extended at all times throughout the experiment. Every 5 minutes after injection the urine was tested for the presence of dye. At its first appearance and every 15 minutes thereafter the amount of dye in 5 ml urine was measured in a Klett colorimeter by the method of Dandy and Rowntree.[6] If its disappearance from the urine was rapid, measurements were taken more often. The bladder was emptied after each sample of urine was withdrawn for a reading. All animals were standardized in the manner just described from one to 15 days before any other compound was administered. All instillations were made into the right hind talocrural articulation. The maximum number of tests in each rabbit was 5.

The doses and channel of administration of each compound used are listed in Table I. Compounds injected directly into the joint cavity were administered along with the dye. All intramuscular and intravenous injections were made 30 to 90 minutes before PSP or hyaluronidase was instilled into the joint cavity. The hyaluronidase used was prepared from bull testes and assayed 8400 turbidity reducing units per mg of nitrogen. The urinary spreading factor was isolated in our laboratory from normal urine and has been shown not to be hyaluronidase. Ten mg of the urinary spreading factor had the same activity as 150 turbidity reducing units of hyaluronidase in causing the spread of dye in the skin of rabbits. Colchicine was administered as the alarming stimulus to 2 rabbits in order to cause endogenous release of adrenal corticoids.

Results. The effects of hyaluronidase and steroids on the permeability of the synovial membrane are shown in Table I. Dye first appeared in the urine of the control animals on an average of 14 minutes after instillation of PSP into the synovial cavity. The urinary concentration of dye at this time was 49 γ per ml. The peak concentration was reached within 30 minutes with an average of 126 γ per ml. Excretion was complete on an average of 40 minutes, at which time the concentration was 3 γ per ml. The ranges of variation for the time of initial appearance and for the initial concentration were surprisingly small. The time to reach the peak, the concentration at 30 minutes, and the duration of excretion showed similar constancy.

1. *Effect of hyaluronidase and of urinary spreading factor.* Instillation of 150 turbidity reducing units of hyaluronidase and PSP into the joint resulted in more rapid absorption and elimination of the dye. The first appearance of dye was in 9 minutes, with a urinary concentration of 119 γ per ml, which was the peak. The duration of excretion was 20 minutes. Intravenous injection of 120,000 turbidity reducing units of hyaluronidase per kg of body weight caused the dye to first appear in the urine in 8 minutes at a concentration of 110 γ per ml, which was the peak. Excretion was completed in 25 minutes. Intravenous injection of 12,000 turbidity reduc-

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[5] Curtis, G. M., and Brunschwig, A., PROC. SOC. EXP. BIOL. AND MED., 1930, **27**, 358.

[6] Dandy, W. E., and Rowntree, L. G., *Ann. Surg.*, 1914, **59**, 587.

TABLE I.

Influence of Hyaluronidase and Steroids on Permeability of Synovial Membrane *in vivo.*

Procedure	No. of rabbits	Min. from instillation of PSP i.j. to its appearance in urine					Gammas PSP in 1 cc of urine					
		First		Peak	Dur 'n of excretion		Onset		Peak		End	
		Avg	Range		Avg	Range	Avg	Range	Avg	Range	Avg	Range
Control	16	14	13–16	30	45	43–48	49	48–50	126	123–127	3	3
150 TRU Hydase* i.j.	6	9	8–10	9	20	19–21	119	118–119	119	118–119	4	4
12,000 TRU Hydase/kg i.v.	2	12	12–13	30	46	46–47	52	52	102	102	4	4
120,000 TRU Hydase/kg i.v.	1	8	—	8	25	—	110	—	110	—	7	—
U.S.F. (non-Hydase)	2	14½	14–15	30	47	—	48	48–49	126	125–127	35	3–4
1 u. A.C.E. i.j.	3	23	23–24	30	86	86–87	30	30–31	68	68	49	49–50 (60 min.)
100 u. A.C.E. i.m.	4	22	19–22	30	139	138–140	40	40–41	49	49	21	21 (130 min.)
1 u. A.C.E. i.j. + Hydase i.j.	3	20	19–20	30	73	72–73	42	42	54	54–55	41	41 (70 min.)
100 u. A.C.E. i.m. + Hydase i.j.	4	17	16–18	30	135	135–136	46	46–47	58	58–59	25	25 (130 min.)
5 mg DOCA i.m.	2	10	10	10	19	19–20	112	112–113	112	112–113	4	4
5 mg DOCA i.m. + Hydase i.j.	2	9	9–10	9	19	19–20	111	111	111	111	5	5
Alarm reaction†	2	More than 2¼ hr					No dye in urine in 2¼ hr All present in joint cavity at autopsy					
Acute adrenalectomy	2	14½	14–15		46	46		Specimens contaminated by hematuria				
Acute adrenalectomy + Hydase i.j.	1	10	—	10	21	—	118	—	118	—	—	4
2 mg Estrone i.m.	2	15	15	15	243	242–244	69	69–70	69	69–70	4	4
2 mg Estrone i.m. + Hydase i.j.	2	19	19–20	19	246	246	70	70	70	70	5	5

i.j. = In talocrural articulation.
TRU = Turbidity reducing units.
i.m. = Intramuscular inj.
PSP = Phenolsulphonphthalein.
USF = Urinary spreading factor.

* Testicular hyaluronidase, Wyeth.
A.C.E. = Adrenal cortical extr. (alcoholic), Upjohn Co.
DOCA = Desoxycorticosterone acetate in oil.
† Induced by 3 mg Colchicine/kg body wt subcutaneously.

ing units of hyaluronidase per kg resulted in no significant change from the normal excretion pattern.

Instillation of urinary spreading factor in an amount equal to the spreading activity of 150 turbidity reducing units of hyaluronidase did not alter the normal excretion pattern.

2. *Effect of adrenal cortical extract* (ACE). Instillation of one unit of ACE into the joint or intramuscular injection of 100 units delayed the initial appearance of dye in the urine to 23 minutes at a concentration of 30 to 40 γ per ml. The peak of 49 to 68 γ per ml was attained within 30 minutes. Excretion was prolonged so that the concentration at 60 minutes was 49 γ per ml and 21 γ in 130 minutes. Instillation of 150 turbidity reducing units of hyaluronidase into the joint of ACE treated rabbits did not significantly alter the excretion pattern obtained with either channel of administration of ACE.

3. *Effect of desoxycorticosterone acetate* (DOCA). Intramuscular injection of DOCA decreased the time for initial appearance of dye in the urine to 10 minutes at a concentration of 112 gammas per ml, which was also the peak. Excretion was completed in 19 to 20 minutes. Instillation of 150 turbidity reducing units of hyaluronidase into the synovial cavity of DOCA treated rabbits did not further hasten absorption and excretion.

4. *Effect of alarm reaction.* PSP was not absorbed from the synovial cavity of rabbits in colchicine alarm reaction, and all the dye could be recovered from the synovial cavity more than two hours after its instillation.

5. *Effect of adrenalectomy.* The pattern of excretion begun 30 minutes after adrenalectomy was essentially the same as in the intact controls. Hyaluronidase had no greater effect in such an adrenalectomized rabbit than in intact rabbits.

6. *Effect of estrone.* Dye first appeared in the urine of estrone treated rabbits in 15 minutes at a concentration of 69 gammas per ml, which was the peak. The duration of excretion was prolonged to 243 minutes. Instillation of 150 turbidity reducing units of hyaluronidase into the joint of estrone treated rabbits did not significantly alter this pattern.

Discussion. The surprising uniformity of rate of absorption from the joints of normal rabbits and the constant effect of hyaluronidase lends great weight to the results obtained when small groups of animals were subjected to the other experimental procedures under test and when small changes were obtained. The more rapid absorption of PSP from the joint of rabbits receiving hyaluronidase is undoubtedly due to increased permeability of the synovial membrane. Presumably this is a specific effect on the hyaluronic acid of this membrane since a non-hyaluronidase spreading factor did not alter its permeability. The large amount of hyaluronidase that was needed by intravenous injection to produce the same effect as a small amount applied locally is accounted for by the presence of hyaluronidase inhibitors in the blood.

The delayed and prolonged absorption of PSP from the joint of rabbits receiving adrenal cortical extract is undoubtedly due to decreased permeability of the synovial membrane. ACE probably altered the state of polymerization of the hyaluronic acid of the membrane since hyaluronidase was no longer effective in increasing its permeability in ACE treated animals. Inhibition of hyaluronidase activity by ACE has been observed in skin[7] and isolated membranes.[1] Estrone treatment also prolonged but did not significantly delay the onset of absorption of PSP. Since hyaluronidase did not increase the permeability of the synovial membrane in the estrone treated rabbits, it is assumed that estrone also altered the hyaluronic acid of the membrane. Increased resistance to hyaluronidase by estrone has been observed in skin[8] and isolated membranes.[1]

The synovial membrane was more permeable to PSP during DOCA treatment than in the control period. The effect was identical with that produced by hyaluronidase. It was maximal and could not be augmented by the instillation of hyaluronidase into the joint. It is significant that the total adrenal steroids had the opposite effect of one of them. The state of permeability of synovial membranes could be governed by the balance between DOCA and some other adrenal steroid. A similar opposition of effect was noted in isolated membranes[1] and in human hypertensives.[9] Furthermore, DOCA aggravates rheumatoid arthritis,[10] while Compound E alleviates this disease.[4]

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[7] Opsahl, J., White, A., and Duran-Reynals, F., *Ann. N. Y. Acad. Sci.*, in press.

[8] Sprunt, D. H., and McDearman, S., *Endocrinology*, 1940, **27**, 893.

The synovial membrane of rabbits in the alarm reaction phase of the adaptation syndrome was completely impermeable to PSP. The dye could be recovered from the joint more than 2 hours after its instillation. Isolated urinary bladder membranes prepared from rabbits in the alarm reaction are impermeable to the passage of fluid when used to separate M/1 sucrose from distilled water, while such bladders prepared from normal rabbits act as semipermeable membranes under the same conditions.[1] Such semipermeable membranes become impermeable to the further passage of fluid if serum from a rabbit in the alarm reaction is added to them.[1]

Adrenal steroids are released during the adaptation syndrome in response to physical, chemical, and psychic stresses. A protective role is attributed to these in the alarm reaction stage.[3,11] Their role during the stage of adaptation is not clear, but the diseases that are thought to arise during this stage are attributed to DOCA intoxication. It might well be that protective steroids possibly of the Compound E type are predominant during the alarm reaction phase and that possibly DOCA types dominate the adaptation phase. Alleviation of adaptation disease could then be obtained by administration of Compound E types or by the administration of adrenocorticotrophic hormone. It is of interest in this respect that DOCA does not affect the blood pressure of normotensive individuals but aggravates hypertension, which is considered to be a disease of adaptation,[3] and that the aggravation is nullified by ACE.

The synovial membrane permeability was not altered shortly after adrenalectomy, nor was the response to hyaluronidase abnormal. Depletion of adrenal steroids could not have occurred in this short period. Changes in permeability will probably be seen in the animals under study at various intervals after adrenalectomy.

The findings reported in this paper may be of particular significance in considering the pathogenesis and treatment of rheumatoid arthritis. This disease appears to be a deficiency in the function of hyaluronic acid of the synoviae. We have demonstrated that similar abnormal function of lowered resistance and increased permeability can be induced by either hyaluronidase or DOCA, both of which could permit exudation into joints. The etiology of rheumatoid arthritis has been attributed to hyaluronidases attacking the synovial structures[2] and to what may be DOCA intoxication due to various stresses.[3] We have furthermore shown that the effect of hyaluronidase is antagonized by ACE and endogenously released adrenal steroids by increasing the resistance of the synovial barrier. We have also demonstrated that DOCA acts in opposition to the other steroids present in ACE. These findings are to be considered in explaining the mechanism of the beneficial effect of Compound E, adrenocorticotrophic hormone, and possibly colchicine and salicylates in the treatment of rheumatoid arthritis and rheumatic fever.[12,13]

Summary and conclusions. 1. The permeability of the synovial membrane as measured by speed of absorption and excretion into the urine of phenolsulphonphthalein (PSP) instilled into the joint was found to be surprisingly constant in a group of 16 normal rabbits.

2. Hyaluronidase markedly increased permeability of the synovial membrane. The effect was maximal and was not augmented by desoxycorticosterone acetate (DOCA).

3. Adrenal cortical extract decreased permeability of the synovial membrane and an-

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[9] Perera, G. A., and Pines, K. L., PROC. SOC. EXP. BIOL. AND MED., 1949, **71**, 443.

[10] Degennes, L., Mahoudeau, D., and Bricaire, H., *Bull. Soc. Med. Hosp. Paris,* 1947, **63**, 532.

[11] Long, C. N. H., *Fed. Proc.,* 1947, **6**, 461.

[12] Forman, C., Seifter, J., and Ehrich, W. E., *J. Allergy,* 1949, **20**, 273.

[13] Hench, P. S., Slocumb, C. H., Barnes, A. R., Smith, H. L., Polley, H. F., and Kendall, E. C., *Proc. Staff Meet., Mayo Clinic,* 1949, **24**, 277.

tagonized the effect of hyaluronidase. This effect of adrenal steroids was more pronounced when they were released endogenously by the alarm reaction. Estrone also decreased the permeability of synovial membrane.

4. Desoxycorticosterone increased maximally the permeability of synovial membrane to the same extent as hyaluronidase and could not be augmented by hyaluronidase.

5. It is suggested that the normal permeability of the synovial membrane is in part controlled by the balance between adrenal steroids of the DOCA type and of the Compound E type.

6. These findings are consistent with current views of the etiology of rheumatoid arthritis either by hyaluronidases or from exposure to stressing stimuli. They are also consistent with the therapeutic effects of Compound E or of adrenocorticotrophic hormone in the treatment of arthritis.

Addendum: Since completion of this work cortisone acetate and adrenocorticotrophic hormone (ACTH) were made available to us. One mg of cortisone acetate per kg and 0.5 mg of ACTH per kg were almost as effective in decreasing permeability of the synovial membrane as the alarm reaction. With both compounds only 25 to 30% of the dye was excreted in 4½ hours. The remainder was recovered from the synovial cavity. On this basis, the dose of adrenal cortical extract and estrone listed in Table I has cortisone activity of less than 20%, and the normal membrane balance of DOCA:cortisone an activity of 4.5%.

Received Sept. 29, 1949. P.S.E.B.M., 1949, **72**.

Development of Adrenal Cortical Adenomas in Ovariectomized Mice Injected with "Physiologic" Doses of Sex Hormone.* (17407)

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Since the development of adrenal cortical adenomas in gonadectomized mice has been inhibited by the implantation of pellets of estrogenic hormone,[1] it might be inferred that withdrawal of gonadal secretion is of primary importance in precipitating the development of the castration-induced adenoma of the mouse adrenal cortex. The objective of the experiments being reported was to determine the effect of the administration of regulated, "physiologic" amounts of estrogenic or androgenic hormone on the induction of adrenal cortical tumors in susceptible ovariectomized mice.

Mice of the NH strain were used in these experiments. NH females develop adrenal cortical tumors spontaneously following ovarian regression, and precociously if gonadectomized.[2] Adrenal cortical adenomas are present in most NH mice of both sexes 5 months after removal of the gonads.

Four NH males and one female which had been gonadectomized at 23 days of age received (by subcutaneous injection beginning 2 weeks after operation) 0.05 microgram of estradiol dipropionate[‡] in sesame oil at intervals of from 5 to 9 days. This treatment with estrogenic hormone provided for constant estrus, as determined by vaginal smear. These

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* This investigation has been aided by grants from the National Cancer Institute, The Jane Coffin Childs Memorial Fund for Medical Research, and the Cancer Fund of the Graduate School of the University of Minnesota.

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[1] Woolley, G., and Little, C. C., *Cancer Research*, 1946, **6**, 491.

[2] Kirschbaum, A., Frantz, M. F., and Williams, W. L., *Cancer Research*, 1946, **6**, 707.

[‡] The synthetic sex hormones were supplied by Ciba Pharmaceutical Products through the courtesy of F. E. Houghton.

5 animals were autopsied 6 months after the institution of hormone treatment. Administration of hormone was suspended 53 days before autopsy in 2 NH females which received similar treatment.

Two ovariectomized NH females received the same treatment with the estrogen and in addition were injected weekly with 150 μg of progesterone in oil. Two additional ovariectomized females received the estrogen plus progesterone, but treatment was suspended 47 days before autopsy.

Two NH females ovariectomized at 21 days of age were given 17.5 μg of testosterone propionate in oil weekly by subcutaneous injection beginning 2 weeks after operation. This treatment was continued until the animals were autopsied 5½ months after ovariectomy. Three mice were similarly treated except that they received no injections of hormone for the 46 days preceding autopsy.

Histologic serial sections of the adrenal glands were studied following complete autopsy. Representative sections of the reproductive tracts, submaxillary glands and kidneys were observed to assay the hormonal status of the experimental animals.

Cortical adenomas appeared in gonadectomized mice of the NH strain receiving amounts of estrogenic hormone just sufficient to maintain constant estrus. Ovariectomized females receiving masculinizing doses of testosterone propionate (as judged by submaxillary gland histology) also developed cortical adenomas. Similar adenomas were present in the ovariectomized females which received estrogen and progesterone simultaneously, and in ovariectomized females receiving no hormone injections. A group of 4 ovariectomized NH females which received 0.1 μg of estradiol dipropionate weekly for 4 months following operation at 35 days of age had definite adenomas at autopsy.

Seventeen of 20 gonadectomized mice on hormone treatment developed adenomas which were in all respects similar to those observed in controls which had been merely gonadectomized. Weekly injections of 0.05 μg of estradiol dipropionate failed to prevent the development of spontaneous adrenal cortical adenomas in NH females.[3]

Since adrenal cortical adenomas developed in gonadectomized mice receiving maintenance doses of sex hormone, it appears that absence of functional gonadal tissue, rather than withdrawal of sex hormone, is the prime factor in the development of adrenal cortical adenomas in gonadectomized mice. The gonad, as compared to the adrenal, may preferentially utilize trophic hormone of the pituitary gland. When functional gonadal tissue is absent in a strain of mice with an extragonadal sensitive target organ, such as the NH adrenal cortex, adenomas develop.

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[3] Frantz, M. F., unpublished data.

Received October 10, 1949. P.S.E.B.M., 1949, **72**.

Search for Antibiotics Active Against *Mycobacterium tuberculosis*. 1. A Streptothricin-like Antibiotic from a *Streptomyces sp.** (17408)

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In a search for antibiotics active against *Mycobacterium tuberculosis* var. *hominis* several hundred antagonists have been encountered and one called *Streptomyces sp.* EI_5 in our records was chosen for intensive study because of its marked activity against the mycobacteria and the low animal toxicity of its crude filtrate.[1] The present report is

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* This investigation was supported in part by the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service, and in part by the Alice McDermott Research Foundation of the University of Washington.

the account of a study of this organism and the antibiotic it produces.

Methods and Results. A. Morphological and Cultural Characteristics of Streptomyces sp. EI_5. Since preliminary tests of solubility and antibacterial activity indicated that the antibiotic produced by *S. sp* EI_5 is similar to streptomycin and streptothricin, a comparison of the organisms that produce these antibiotics was made.

Streptomyces sp. EI_5 was morphologically similar to *Streptomyces griseus,* and both organisms produced a soluble yellow to yellow-brown pigment when grown at room temperature on nutrient agar, glucose agar, starch agar, and gelatin. Only *S. griseus* produced pig. ment on potato agar. The color of the aerial mycelium varied from white to tea-green with *S. griseus* and from white to gray with *S. sp.* EI_5. The vegetative mycelium of *S. griseus* was white to cream colored and that of *S. sp.* EI_5 yellow to tannish orange. *Streptomyces sp.* EI_5 was not susceptible to the *S. griseus* actinophage of Reilly, Harris and Waksman.[2] These observations indicate that *S. griseus* and *S. sp.* EI_5, are similar but significantly different organisms.

Streptomyces lavendulae produced a darker brown pigment on glucose agar, nutrient agar and glucose broth than *S. sp.* EI_5, and in addition produced pigment on the potato plug. Further, slide cultures on Czapek's medium at 30°C showed the aerial mycelium of *S. lavendulae* to be pink to lavender, and the hyphae often coiled terminally, in contrast to the white to gray color and the straight hyphae of *S. sp.* EI_5. The conidia of *S. sp.* EI_5 were observed to be oval to globose and considerably smaller than those of *S. lavendulae.* These differences indicate that *S. lavendulae* and *S. sp.* EI_5 are not identical.

It is also probable that *S. sp.* EI_5 is distinct from the organism described by Trussell, Fulton and Grant[3] since, unlike our organism, their organism produces streptomycin and displays tightly coiled terminal hyphae. In the latter respect, our organism differs also from the two *Actinomyces sp.* described by Kelner and Morton[4] (A-10 and A-105) which produce the antibiotics, lavendulin and actinorubin.

B. *Production of Antibiotic* EI_5.‡ The 1% glucose medium of Waksman and Woodruff[5] proved to be as satisfactory as any medium tried including modifications of the soybean medium described by Rake and Donovick.[6]

Attempts to enhance antibiotic production by the procedure employed for *S. griseus* by Waksman, Reilly, and Johnstone[7] of growing the organism in high concentrations of its own antibiotic failed. The standard inoculum adopted was a heavy spore suspension of a high antibiotic-producing substrain. The organisms were grown in shake cultures for 5-7 days and the culture fluid harvested when the pH rose to 7.0 to 7.4. It was filtered through cotton and stored at –45°C prior to extraction.

C. *Concentration and Purification of* EI_5. Since the antibiotic was found to belong to the 3rd solubility group of Waksman,[8] the general scheme of the procedure used by LePage and Campbell[9] for the concentration of streptomycin was adopted. It consisted of adsorption on carbon, elution with acid methanol and precipitation with acetone. The total yield in this concentrate was approximately 30% of the amount present in the original filtrate.

Further purification was accomplished by the use of the synthetic zeolite Decalso. Spot plate analyses by the method of Loo and co-

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[3] Trussell, P. C., Fulton, C. O., and Grant, G. A., *J. Bact.*, 1947, **53**, 769.

[4] Kelner, A., and Morton, H. E., *J. Bact.*, 1947, **53**, 695.

‡ Antibiotic EI_5 is subsequently designated as EI_5.

[5] Waksman, S. A., and Woodruff, H. B., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 207.

[6] Rake, G., and Donovick, R., *J. Bact.*, 1946, **52**, 223.

[7] Waksman, S. A., Reilly, H. C., and Johnstone, D. C., *J. Bact.*, 1946, **52**, 393.

[8] Waksman, S. A., Microbial Antagonisms and Antibiotic Substances, p. 171, The Commonwealth Fund, New York, N. Y., 1945.

[9] Le Page, G. A., and Campbell, E., *J. Biol. Chem.*, 1946, **162**, 163.

workers[10] showed that most of the antibiotic was adsorbed on the Decalso column and all dark pigment eliminated.

The eluate was evaporated *in vacuo* to a small volume and the antibiotic brought down as a fine white precipitate by addition of acetone. Still further purification was carried out by the chromatographic method as employed by Carter and co-workers[11] for the purification of streptomycin.

The material was finally concentrated *in vacuo,* reprecipitated with acetone, dissolved in double glass-distilled water and sterilized by passage through a sintered glass filter.

D. *Methods of Assaying* EI_5. The potency of EI_5 was determined by a serial dilution method employing the broth base of the medium recommended by Loo and co-workers[10] for streptomycin assay. The unitage is expressed as dilution units using *Escherichia coli-communior* as the test organism. On the basis of dry weight, the final product was bacteriostatic for *E. coli-communior* in a dilution of approximately 1:200,000. The activity of various batches of antibiotic was observed to vary considerably and no consistent results were obtained which would make possible an accurate comparison of the effectiveness of the several purification methods used.

E. *Antibacterial Spectrum of* EI_5. Antibacterial tests readily established that EI_5 and streptomycin are different antibiotics. For example, the minimum concentrations inhibitive for *Bacillus subtilis* were EI_5, 1.6 units and streptomycin, 4.0 units. With *Serratia marcescens* the values were EI_5 30.0 units and streptomycin, 0.75 unit.

The differences between the antibacterial spectra of EI_5 and streptothricin were less marked.

In a further attempt to identify EI_5 resort was made to the development of strains of test organisms resistant to streptothricin and to EI_5. The parent strains of organisms used for this work were *B. subtilis, E. coli* M3G and *Micrococcus pyogenes var, aureus* 209-P. They were cultured at 37°C in nutrient broth pH 7.1, containing varying amounts of antibiotic covering the ranges of tolerance and subcultures made at 24-hour intervals with inocula from the highest concentration of antibiotic showing growth in the previous series.

Sixty or more serial transfers were made over a period of 3 months before cultures of appreciable resistance were obtained (cf. Kelner and Morton,[4] and Trussell, Fulton, and Grant[3]). It was noted that some of the organisms tended to lose their acquired resistance upon repeated subculture on nutrient agar.

The antibacterial spectra of streptothricin and purified EI_5, determined with the parent and resistant strains shown in Table I, clearly indicate that streptothricin and EI_5 are different substances. It is noteworthy that all of the resistant strains developed against EI_5 also showed marked resistance to streptothricin, whereas, resistant strains developed against streptothricin do not show as uniform and marked increase in resistance to EI_5. Of additional interest is the case of *B. subtilis* cultivated in EI_5 in which a marked resistance to streptothricin resulted without an increase in resistance to EI_5.

One explanation which may account for these findings is that the development of resistance to one antibiotic may result in the development of resistance to another closely related antibiotic (cf. Kelner and Morton[4]). Another possible explanation is that the preparation of EI_5 may contain a small amount of streptothricin as an impurity.

F. *Comparison of* EI_5 *with other Streptothricin-like Antibiotics.* Since lavendulin and actinorubin (Kelner and Morton[4]) were not available, resort was made to cross-streak antibacterial spectral analysis of *S. sp.* EI_5, *S. sp.* A-10, and *S. sp.* A-105 in an attempt to obtain information on the identity of the respective antibiotics. The results are presented in Table II.

The marked variation in the zones of inhibition shown by *S. sp.* A-10 and *S. sp.* EI_5 indicates strongly that the antibiotic produced

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[10] Loo, Y. H., Skell, P. S., Thornberry, H. H., Ehrlich, J., McGuire, J. M., Savage, G. M., and Sylvester, J. C., *J. Bact.*, 1945, **50**, 701.

[11] Carter, H. E., Clark, R. K., Jr., Dickmans, S. R., Loo, Y. H., Skell, P. S., and Strong, W. A., *J. Biol. Chem.*, 1945, **160**, 337.

TABLE I.
Antibacterial Spectra of Streptothricin and EI_5 Determined with Resistant and Parent Strains of Bacteria.
(Readings taken after 24 hr at 37°C in nutrient broth at pH 7.0).

Test organism	Min. inhibiting conc. of streptothricin in dilution units	Min. inhibiting conc. of EI_5 in dilution units
Bacillus subtilis (parent)	12	4
” ” (streptothricin resistant)	120	8
” ” (EI_5 resistant)	90	4
Escherichia coli (parent)	0.6	0.8
” ” (streptothricin resistant)	8	8
” ” (EI_5 resistant)	20	20
Micrococcus aureus (parent)	0.3	0.5
” ” (streptothricin resistant)	22	4
” ” (EI_5 resistant)	36	40

TABLE II.
Cross-streak of Antibacterial Spectra of *Streptomyces sp.* A-10, *Streptomyces sp.* EI_5, and *Streptomyces sp.* A-105.

Test organism	Zone of inhibition in mm		
	S. sp. A-10	*S. sp.* EI_5	*S. sp.* A-105
Achromobacter sp.	18	16	12
Aerobacter aerogenes	20	30	25
Bacillus cereus	10	3	5
” *megatherium*	17	15	10
” *mycoides*	25	30	30
” *sp. 290*	22	27	20
” *subtilis*	8	1	3
Corynebacterium xerose	12	2	7
Escherichia coli-communior	20	20	15
Micrococcus aureus 209-P	18	15	15
Sarcina lutea	20	15	10
Serratia marcescens	12	2	2
EI_5 resistant			
Bacillus subtilis	8	5	5
Micrococcus aureus	10	3	5
Streptothricin resistant			
Bacillus subtilis	5	1	1
Escherichia coli-communior	15	7	7
Micrococcus aureus	25	7	12

by *S. sp.* A-10 (lavendulin) is different from EI_5. In contrast, the zones of inhibition shown by *S. sp.* A-105 and *S. sp.* EI_5 are similar indicating that a close relationship between actinorubin and EI_5. Further both substances are soluble in water and acid-methanol, insoluble in ether and acetone, resist boiling, and, as shown later, have similar toxicities for mice.

G. *The Antibacterial Activity of Antibiotic EI_5 for the Mycobacteria.* By use of the method described by Youmans[12] EI_5 was shown to be bacteriostatic for *M. tuberculosis* H37Rv in a dilution of 1:100,000 and for *Mycobacterium phlei* in a dilution of 1:500,000.

H. *Toxicity of EI_5 for Mice.* Large doses of EI_5 ranging from 150,000 to 270,000 units per kg in the form of both the initial concentrate and purified antibiotic were injected intraperitoneally into 10 white mice weighing 20 to 35 g. One animal died on the 5th day and the others were sacrificed from the 5th to the 19th day. The results demonstrated that EI_5 is apparently less toxic than streptothricin as judged by the findings of Molitor,[13] and more nearly equal to the toxicity of actinorubin as determined by Morton.[14] Both actinorubin and EI_5 in large dosage cause focal necrosis of the liver and renal epithelial damage.

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[12] Youmans G. P., Proc. Soc. Exp. Biol. and Med., 1944, **57**, 119.

Summary. Search for antibiotics active against *Mycobacterium tuberculosis* var. *hominis* has resulted in the isolation of a streptothricin-like antibiotic, termed EI_5, from an unidentified *Streptomyces sp.* It is bacteriostatic for *Mycobacterium tuberculosis* H37Rv in dilution of 1:100,000 and closely resembles actinorubin in its antibacterial spectrum, general properties, and toxicity.

The organisms that produce the two antibiotics, *Streptomyces sp.* A-105 and *Streptomyces sp.* EI_5 are morphologically distinct.

The authors gratefully acknowledge the help of, and are indebted to Dr. Selman A. Waksman of Rutgers University for the cultures of *Streptomyces griseus* and actinophage; Merck and Company, who kindly furnished the streptomycin and streptothricin used in the experiments; Dr. H. B. Woodruff of the Merck Laboratories for the culture of *Escherichia coli* M3G; Dr. Dorothy Hamre of the Squibb Institute for Medical Research for the cultures of *Micrococcus aureus* 209-P and *bacillus* No. 290; Dr. Harry E. Morton of the University of Pennsylvania for the cultures of *Streptomyces sp.* A-10 and *Streptomyces sp.* A-105.

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Received October 11, 1948. P.S.E.B.M., 1949, **72**.

Multiplicity of Antigens in Ragweed Pollen Extracts as Demonstrated by the Technic of Oudin. (17409)

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The heterogeneity of ragweed pollen extracts has been demonstrated by chemical fractionation[1] and by electrophoretic analysis.[2] Precipitins to crude extracts of ragweed pollen have been produced by numerous workers,[3,4] but because of the heterogeneity of the extracts used, it would be expected that the precipitins so obtained were directed to a number of different antigenic substances. It is the purpose of this paper to show, by a new technic, the presence of a multiplicity of antibodies in the antisera produced against crude extracts of ragweed pollen, and consequently, the presence of a multiplicity of antigens in the extracts used for immunization.

The new technic used to demonstrate this antigenic complexity was that recently described by Oudin[5-9] which consists in overlaying a solid immune serum-agar mixture contained in a thin-bore tube with the antigen solution. An antigen-antibody precipitate is formed as a sharp band which moves down the tube as more and more antigen diffuses into the agar. Oudin showed that only one band is formed when a single antigen-antibody system is present, while multiple systems give

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[2] Abramson, H. A., *Ann. Allergy*, 1947, **5**, 19.

[3] Kukla, A., and Hirsch, D., *J. Immunol.*, 1946, **53**, 391.

[4] Eagle, H., Arbesman, C. E., and Winkenwerder, W. L., *J. Immunol.*, 1939, **36**, 425.

[5] Oudin, J., *Compt. Rend. Acad. Sci.*, 1946, **222**, 115.

[6] Oudin, J., *Bull. Soc. Chim. Biol.*, 1947, **29**, 140.

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[8] Oudin, J., *Annal. de l'Inst. Pasteur*, 1948, **75**, 109.

[9] Oudin, J., *Compt. Rend. Acad. Sci.*, 1949, **228**, 1890.

TABLE I.
Supernatant Tests on Ragweed Pollen Extract and Its Homologous Rabbit Antiserum.

Tube No.	Ragweed extract, ml	Precipitable N in mg per ml of antiserum	Precipitin test on supernatant (ring method) Presence of: Antigen	Antibody
1	.04	.060	—	+
2	.08	.086	—	+
3	.12	.120	—	+
4	.16	.126	—	+
5	.20	.140	±	+
6	.30	.156	+	+
7	.40	.166	+	+

more than one band. The number of such bands corresponds to the minimum number of antigen and antibody systems present. Before applying this technic to ragweed pollen extracts the findings of Oudin were confirmed using crystalline antigens and antisera of known purity. This latter work will be published elsewhere.

Materials and methods. Defatted short ragweed (Ambrosia elatior) pollen was extracted with distilled water or physiological saline in the proportions of 5 g per 100 cc (5% extract) or 10 g per 100 cc (10% extract). The water or saline used in the extraction contained 1:5000 merthiolate (Lilly). The rabbits were immunized by repeated subcutaneous injections of the ragweed pollen extract using Freund's adjuvant technic.[8,10] Before use in the Oudin test the extracts were brought to pH 6.5-7.5 with 0.1N sodium hydroxide, and any precipitate which formed was filtered off. The procedure followed in the Oudin technic was essentially that described by him[7] with only minor modifications. One part of 0.6% Bacto agar (Difco) in physiological saline containing 1:5000 merthiolate was used to mix with one part of antiserum. The agar was kept at 48-50°C and the serum was brought to that temperature before mixing thoroughly with the agar. Precipitin tubes of 3 mm inside diameter and approximately 5-6 cm long, which had been previously coated with 1% agar and dried under vacuum, were filled ½ to 2/3 full with the serum-agar mixture. The tubes containing the serum-agar were then placed in the refrigerator for 5 to 10 minutes to allow the agar to solidify. The antigen solution containing 1:10,000 merthiolate was now layered on top of the solid serum-agar mixture, and the tubes were sealed with plasticine to prevent evaporation and kept for 2 weeks at approximately 25°C.

Results. Quantitative precipitin tests were set up using rabbit antiserum and short ragweed pollen extract in 50% glycerin in the same manner as reported by Bukantz *et al.*[11] The results of the supernatant analysis shown in Table I demonstrate that more than one antigen-antibody system exists, since both antigen and antibody were found in the same supernatant. (See Kendall.[12]) The Oudin test was run using a 10% extract of ragweed pollen in physiological saline. The final concentration of antiserum in the serum-agar mixtures was 50%. An antiserum, in a 50% final concentration, when tested with 5% water extract of the same batch of pollen gave 5 bands. The position of each individual band was measured at 6-18 hour intervals with calipers accurate to 0.1 mm. The results of plotting the distance moved by each of the bands against the square root of the time gave, as found by Oudin with other systems, straight lines (Fig. 1). These experiments were repeated using different antisera with similar results. In some experiments there was an indication of the presence of a sixth band, but this was so faint that one could not be certain of its actual existence.

Discussion. The results of these experiments clearly demonstrate that there are at

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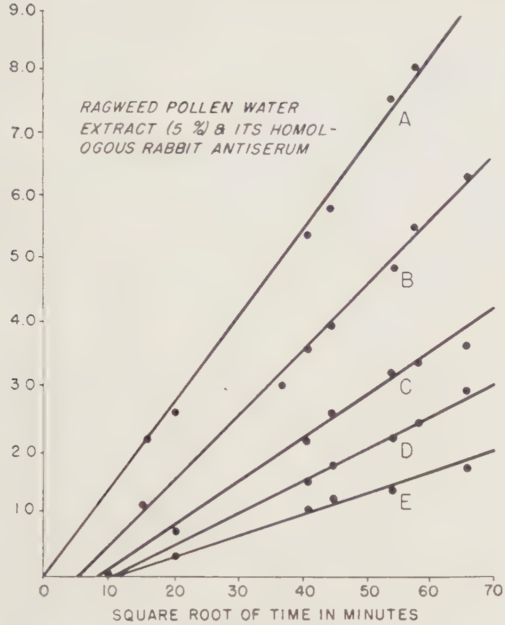

FIG. 1.

Movement of 5 bands of precipitate obtained with ragweed pollen extract and its homologous antiserum plotted against the square root of the time.

least 5 distinct substances in ragweed pollen extracts which are antigenic for rabbits. That the antigenic complexity may be even greater than this is indicated by the observation that complete absorption of the ragweed extract with rabbit antiserum markedly reduces the direct skin reactivity in specifically sensitive human beings, but does not abolish it.[13] The relationship of these 5 antigens to the various chemical[1] and electrophoretic[2] fractions shown to exist in ragweed pollen extracts is not known at present, and more work is needed to elucidate this problem. The technic of Oudin shows great promise in the fractionation of antigenic mixtures. It has been applied by Oudin to the fractionation of horse serum[6] and by us to demonstrate the presence of at least 3 antigens in cytochrome c of 0.34% iron content.[14] Moreover, work from Oudin's laboratories[9] and from our own (unpublished experiments) indicates that it may be possible to apply his method as a quantitative tool for the determination of either antigen or antibody concentrations.

Conclusion. Application of the technic of Oudin to ragweed pollen extracts and their antisera demonstrated the presence of at least 5 different antigens in such extracts.

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Received June 15, 1949. P.S.E.B.M., 1949, **72**.

Allergic Encephalitis Following Injection of Dialysate of Brain Tissue.
(17410)

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(Introduced by K. Habel)

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The encephalitic process which follows the injection into monkeys[1,2] and guinea pigs[3,4] of homologous or heterologous brain tissue has been variously termed meningo-encephalomyelitis,[7] acute disseminated encephalomyelitis,[1] isoallergic encephalomyelitis[3] or allergic encephalitis,[5] and has been compared with the post-vaccinal (rabies) paralysis of human beings.[10] The nature of the factor in brain which is responsible for this process is not known, although reports have appeared showing that the factor is present in the phosphatide fraction of brain[7,11] and in the protein fraction remaining after extraction of all brain lipoids.[11] Work on the purification of rabies vaccine[5] has shown that the factor which causes allergic encephalitis is soluble in a solution of calcium acetate. This factor was separated from rabies vaccine by washing the infected brain tissue carefully with calcium acetate solution after extraction with benzene and ether. When incorporated into the mineral oil adjuvant described by Freund and McDermott,[6] the separated factor produced allergic encephalitis in guinea pigs. This report describes some observations made during a study of the properties of the factor causing allergic encephalitis.

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* Laboratory of Infectious Diseases.

† Laboratory of Pathology.

‡ Died May 14, 1949. Laboratory of Biologics Control.

[1] Kabat, E. A., Wolf, A., and Bezer, A. E., *J. Exp. Med.*, 1947, **85**, 117.

[2] Morgan, I. M., *J. Exp. Med.*, 1947, **85**, 131.

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[4] Kopeloff, L. M., and Kopeloff, N., *J. Immunol.*, 1947, **57**, 229.

[5] Bell, J. F., Wright, J. T., and Habel, K., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 457.

The procedure by which dialysates of brain tissue were prepared is an extension of the method[5] for freeing rabies vaccine from the factor causing allergic encephalitis. Brain tissues from rabbits and cattle were minced in a Waring Blendor, suspended in distilled water, and the suspensions dried from the frozen state. The dried brain was extracted with benzene followed by two extractions with ether. After removal of the ether, the brain tissue was suspended in distilled water. When the suspension had become thoroughly hydrated, it was further diluted with an equal volume of M/5 calcium acetate solution, or in some experiments with distilled water. The mixture was placed in cellophane tubing and suspended in 4 volumes of distilled water. All cellophane tubes were tested by filling them with water and observing for leaks while moderate pressure was applied to the top of the tubes. Care was taken to wash the end of the tubing through which brain suspension was introduced so that the dialysate would not be contaminated. After storage at 6° C for 3 to 4 days with frequent agitation, the clear dialysate, which now had a slightly amber tint, was removed and dried from the frozen state. This dialysate usually contained 2 to 4 mg of solids per milliliter at a concentration equivalent to an original 10% brain emulsion.

The presence of the allergic factor in the brain dialysate was determined by mixing a 5 ml sample with 5 ml of the adjuvant (4.5 ml of mineral oil containing 9 mg of dried acid fast bacilli [B.C.G.], plus 0.5 ml Arlacel A) and injecting 1 ml of the mixture subcutaneously into each 200-250 g guinea pig. The animals were observed for at least a month following injection. Those which developed only slight weakness in the hind quarters are recorded as having doubtful symptoms, those with definite weakness or paralysis as positive. Forty-six of the 74 animals used were killed for histological study in various stages of the disease, after clinical recovery, or after 30 to 60 days had elapsed without clinical evidence of allergic encephalitis. Therefore, final mortality figures could not be tabulated. Brains and spinal cords were fixed in formalin and sections through the thalamus, pons, and several levels of the cord were examined. The lesions found were similar in all respects to those previously produced by the injection of unmodified brain tissue in adjuvant. They consisted of scattered, perivascular cuffs of mononuclear and microglial cells about venules in the cerebral white matter, hippocampus, thalamus, pons, cerebellar white matter, and cord. The size and number of the lesions varied greatly in individual animals.

Brain preparations which produced either positive clinical signs or characteristic microscopic brain lesions were listed as containing the factor. In most cases a positive histopathological diagnosis was related to positive clinical signs. In a few cases characteristic lesions were found in animals which showed no clinical signs of the disease. The results of 6 experiments on dialysis are summarized in the accompanying table.

It will be noted that animals injected with the brain preparations either before or after dialysis showed evidence of allergic encepha-

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TABLE I.
Allergic Encephalitis Produced in Guinea Pigs by Various Fractions of Brain Tissue.

Exp. No.	Material*	Amt inj.,† mg	Diagnosis‡ Clinical Positive	Diagnosis‡ Clinical Doubtful	Diagnosis‡ Histopathological Positive
216-A	Cal acetate extr. of normal rabbit brain before dialysis	50	2/6	2/6	1/6
B	Dialysate of normal rabbit brain	50	9/20	8/20	12/18
254	Dialysate of normal rabbit brain	40	3/5	0/5	3/3
241	(Same)	25	2/10	2/10	2/2
233-A	Water extr. of normal beef brain before dialysis	100	6/9	2/9	1/3
251-B	Dialysate of normal beef brain	250	2/10	2/10	5/10
233-B	(Same)	100	2/9	2/9	1/3
D	(Same)	50	1/5	0/5	1/1

* All tissues were previously dried and extracted with benzene and ether.
† Expressed as equivalent weight of wet whole brain.
‡ Denominator of fraction indicates number of animals in each experiment.

litis. In no instance, however, did all of the animals injected with any one preparation develop the characteristic signs or lesions of the disease. Since the incidence of this disease following one dose of whole brain in adjuvant has been variable[4] no attempt was made to determine the amount of factor present by the incidence of positive reactions in animals. All observations which showed a positive picture of allergic encephalitis are regarded as significant.

On the basis of the above it is evident that the substance which causes allergic encephalitis is capable of passing through a cellophane membrane and, therefore, has a molecular weight of approximately 10,000 or less. It will be recalled that Alvord[7] found the factor to be a heat-stable, phosphatide-like substance which might possibly resemble the alcohol-soluble brain-hapten of Lewis[8] and Rudy.[9] Very little is known regarding the properties of the materials recovered in the dialysate, except that the mixture contains salts, amino acids and other substances and is Biuret negative at a concentration equivalent to 200% brain tissue, *i.e.*, a solution with 4 to 8% total solids.

Summary. The dialysate of benzene-ether extracted brain tissue is capable of causing signs and lesions of allergic encephalitis in guinea pigs. The material recovered in the dialysate is free of protein and presumably contains only substances of low molecular weight.

Received Sept. 20, 1949. P.S.E.B.M., 1949, **72**.

Effect of Rose Bengal and Ultra Violet Light on Porphyrin Excretion in Rabbits.* (17411)

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The photodynamic effect of porphyrins *in vitro* and *in vivo* is well known,[1] as is the relationship of sunlight to so called photosensitive or congenital porphyria. In 10 cases of *Pemphigus foliaceus* characterized by photosensitivity, the author has also found large increases in excretion of urinary coproporphyrin.‡ The question arose as to whether a heightened urinary porphyrin excretion might be the effect of photodynamic activity, rather than, as always hitherto supposed, that the excessive porphyrin was the cause of this activity. The well known occurrence of marked increases of urinary coproporphyrin III in a variety of disease states,[2] *without* photosensitivity, in fact suggested that photodynamic activity in the *Pemphigus foliaceus* cases might be causing the coproporphyrinuria.

Rose Bengal is also known to exert a powerful photodynamic action.[3] In studying the fundamental relationship of light to porphyrin excretion the effect of previous sensitization by Rose Bengal and other photosensitizing compounds was investigated. Some of the results are the basis for the present report.

Methods. Adult rabbits of either sex were injected intravenously with a dose of 100 mg of Rose Bengal per kilo of body weight. The dye was dissolved in 10 cc of physiological saline solution.

Unfiltered light from a CH-4 Mercury arc lamp§ of maximum near ultraviolet intensity at 3650 Å, was used for the photosensitization. This wave length corresponds with the maximum absorption of the dye. The hair was shaved from the animal's back and the shaved area was irradiated immediately following the administration of Rose Bengal for 25 minutes at a distance of 12 inches, the approximate focal distance of the lamp.

The concentration of total urinary coproporphyrin was determined with a Lumetron fluorophotometer, in other respects following the technic of Schwartz, Hawkinson, Cohen, and Watson.[4] Their method of isomer analysis[4] was also employed. Crystallization of coproporphyrin methyl ester was done according to methods used previously in this laboratory.[5]

Results. The results are given in Table I. The zero value in each instance serves as a control. Rabbits R1-R5 received both Rose Bengal and ultraviolet light at the beginning of day number 1. Control Rabbit No. 1 was injected with Rose Bengal in the same concentration, but the animal was kept in darkness. Control Rabbit No. 2 received ultraviolet irradiation but no Rose Bengal.

Isomer analysis of the coproporphyrin from Rabbit 1 for the first 24 hours after the photosensitization, revealed 96% coproporphyrin type III, indicating that most of the increase was of this isomer. The methyl ester crystallized in typical rosettes melting at 145°, thus confirming the isomer analysis.

The fecal coproporphyrin was determined during the first 4-day periods in 3 rabbits. No increased values were found.

To control the factor of hemolysis, distilled water was injected intravenously in sufficient

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* Aided by Research Grant No. 345, U. S. Public Health Service.

† Fellow of the Guggenheim Foundation. (From the Instituto Oswaldo Cruz, Rio de Janeiro, Brazil).

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§ Black Light Products, Inc., Chicago, Ill., Model No. B1-2.

[4] Schwartz, S., Hawkinson, V., Cohen, S., and Watson, C. J., *J. Biol. Chem.*, 1947, **168**, 133.

[5] Grinstein, M., Schwartz, S., and Watson, C. J., *J. Biol. Chem.*, 1945, **157**, 323.

TABLE I.
Effect of Rose Bengal and U.V. Light Alone and in Combination on Urinary Coproporphyrin Excretion in Rabbits (γ per 24 hr).

Day	R1	R2	R3	R4	R5	Control 1	Control 2
0	7.0	6.2	6.4	9.7	5.8	7.8	6.6
			Rose Bengal and U.V. light				
1	300.0	110.0	120.0	150.0	135.0	8.6	5.2
2	80.0	96.6	53.2	55.0	76.8	14.0	6.3
3	70.0	70.0	25.0	38.6	62.8	9.0	7.2
4	30.0	12.0	15.0	38.7	15.7	6.8	7.4
5	7.2	10.0	10.0	7.7	13.8	9.4	6.8
6	9.4	5.0	—	15.3	9.7	8.6	8.1
7	10.0	6.3	15.6	21.3	9.8	6.5	7.3
8	10.4	7.8	12.0	16.7	10.1	5.8	6.5
9	10.1	9.4	11.0	16.5	8.7	7.6	7.3

TABLE II.
Effect of Hemolysis Produced by Distilled Water or Phenylhydrazine Hydrochloride on Urinary Coproporphyrin Excretion in Rabbits.

	Urinary Coproporphyrin in γ in 24 hr		
Day	R6	R7	R8
0	7.3	9.7	10.0
	Distilled water	Phenylhydrazine alone	Phenylhydrazine with U.V. light
1	4.1	10.0	9.9
2	9.7	12.0	10.0
3	11.0	16.0	4.5
4	10.8	27.0	10.9
5	9.7	28.0	28.0
6	8.4	26.1	42.0
7		40.7	24.0

quantity to cause a rapid and intense hemolysis in one rabbit. As seen in Table II, there was little if any effect on the coproporphyrin excretion.

For the same purpose 30 mg of phenylhydrazine hydrochloride per kg were injected subcutaneously into 2 rabbits. One of these animals (R8) was exposed to U.V. light for 25 minutes immediately after phenylhydrazine administration.

The mild delayed increase noted in the phenylhydrazine experiments in Table II is probably related to increased erythropoietic activity, as previously found by Schwartz and co-workers.[6]

Summary and conclusions. The administration of Rose Bengal and ultraviolet irradiation in rabbits results in rapid and marked increase in the excretion of urinary coproporphyrin Type III to values 17 to 40 times those in the controls, returning to normal by the 5th day. Neither the dye alone nor the irradiation alone had any effect.

Simple hemolysis by distilled water had no effect upon the urinary coproporphyrin excretion. Phenylhydrazine produced a slight delayed increase of coproporphyrin excretion believed to be due largely to an increase of coproporphyrin Type I, as a result of increased erythropoiesis.

Additional studies are being undertaken in an attempt to discover the origin of the coproporphyrin excreted and the effects of other photodynamic compounds.

These results constitute the first demonstration that photodynamic activity causes a marked increase in the excretion of coproporphyrin III.

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Received July 25, 1949. P.S.E.B.M., 1949, **72**.

Serum Polysaccharide Levels in Experimental Inflammation.* (17412)

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The serum polysaccharide level has been shown to be elevated in malignancies, tuberculosis, and certain other pathological conditions. It has been suggested by Seibert, Seibert, Atno, and Campbell[1] that this rise is related to tissue destruction. They note that the α_2 globulin, which is especially rich in carbohydrate content, is elevated in tuberculosis and carcinomas. Since Shedlovsky and Scudder[2] have noted that a rise in α_2 globulin is associated with inflammation or tissue destruction irrespective of cause, one might postulate that a rise in serum polysaccharides is to be expected wherever inflammation occurs. In studies of human conditions this has, in general, proved to be true,[3] although low results were found in some infectious diseases. It appeared that this problem might be further elucidated by a study of inflammation in experimental animals.

Experimental. Chemical methods. In this study non-glucosamine polysaccharide and protein were determined as previously described.[4] Glucosamine polysaccharide was also determined on all samples, but as the results roughly parallel those for non-glucosamine polysaccharide, they are not given in this paper.

Inflammation due to infection. In order to produce an infectious lesion which could be closely followed, 5 ml of a mixed culture of *Staphylococcus aureus* and *Streptococcus pyogenes* and mucin† were injected subcutaneously into the back legs of healthy mongrel canines. In several cases, large abscesses about 10 cm in diameter resulted. The abscesses usually ruptured spontaneously and drained on about the sixth day; the skin sloughed on the eighth day, leaving the abscess area lined with granulation tissue. The wounds granulated completely within 60 days. The temperature reached a maximal elevation on the third day, but did not return to normal until the sixth day. Clinically, there was no sign of septicemia, and the animals appeared greatly improved by the sixth day. The non-glucosamine polysaccharide level was elevated slightly on the first day, when expressed as a percentage of protein, but did not reach a maximum until the sixth day. The level then fell slowly, but it was still elevated appreciably on the 26th day and did not become normal until the 52nd day. Results obtained from the study of 3 dogs are depicted graphically in Fig. 1. Similar results were not obtained with all dogs, as some animals exhibited more resistance to the infectious agent and no apparent abscess was formed. In these cases the maximal polysaccharide level was not so high, and the return to normal occurred much earlier.

Turpentine Inflammation. Turpentine was sterilized for injection by filtering National Formulary grade turpentine through a Seitz filter by suction into a filter flask. The flask and filter were previously sterilized by auto-

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* This work was supported by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council and by funds from the John Archer Hatchett Memorial Fund.

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† The authors wish to gratefully acknowledge the aid of Dr. Florene Kelly and Dr. Homer Marsh, Department of Bacteriology, Oklahoma University School of Medicine, for their advice in planning the bacterial phase of this work and for their preparation of the culture used.

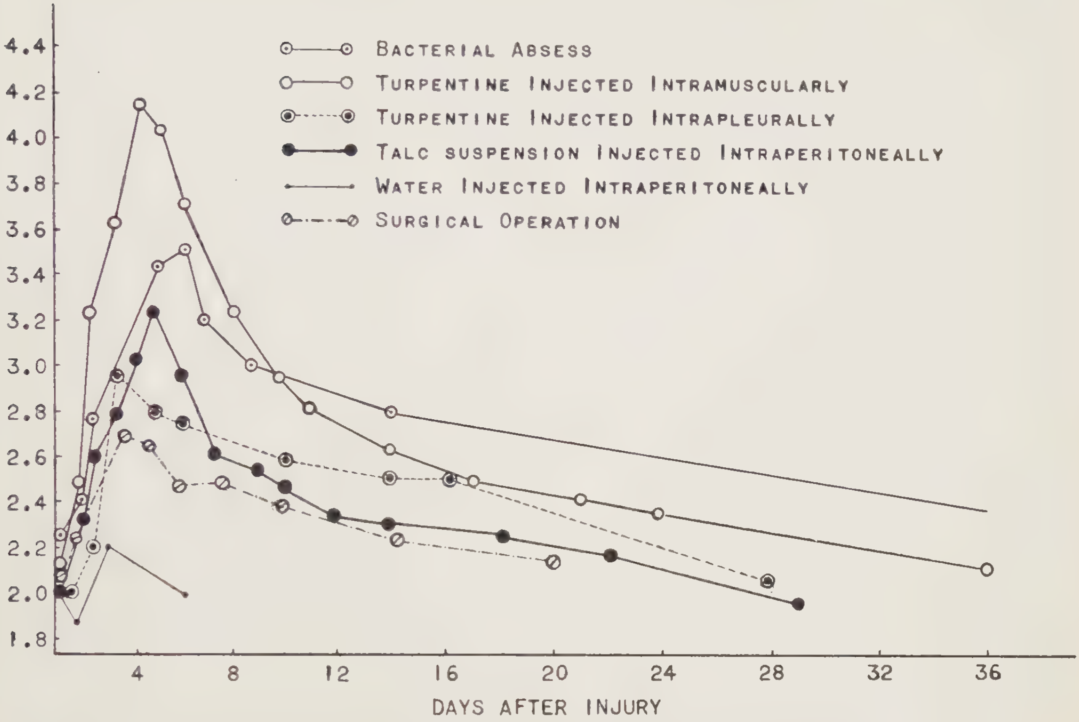

FIG. I. Non-Glucosamine Serum Polysaccharide of Dogs Expressed as a Percentage of Serum Protein Following Injury.

claving for 30 minutes at 15 lb pressure. The turpentine was then transferred to a syringe and injected into the experimental animals, using aseptic technic. In one series of studies the turpentine was injected into the pleural cavity, and in a second series it was injected intramuscularly. Opie[5] describes the former type of injection as "resulting in serofibrinous inflammation which undergoes resolution, so that the pleural cavity is restored to normal in about 10 days; there is no destruction of tissue."

Intramuscular injection of turpentine on the other hand results in a sterile abscess which is filled with purulent material. In this case there is much destruction of tissue early in the course and repair by granulation and fibrosis after evacuation or absorption of the contents of the abscess. In our experiments, using mongrel dogs, swelling usually became apparent after 24 hours, reached a maximum at 48-72 hours, and was reduced after 5 days. The temperature was usually elevated after 24 hours, reached a maximum within 48 hours, and became normal by the fifth day. Abscess formation was noted in every case; in approximately one-half of the experiments the abscess ruptured spontaneously and drained about the fourth day. In these cases it was apparent that considerable tissue damage resulted; the formation of some repair tissue had taken place by the fourth day, when rupture occurred. Average results of the polysaccharide expressed as percentage of protein for 6 animals are expressed graphically in Fig. 1. In both types of injection the serum polysaccharide levels reached maximal elevation on the fourth day, however, the animals injected intramuscularly exhibited the higher maxima.

Talc Granuloma. A talc suspension for injection was prepared by suspending 20 g of talc in 80 ml of water. The suspension was sterilized, allowed to cool, resuspended and injected intraperitoneally into healthy mongrel dogs through a No. 15 needle, using aseptic technic. The signs of peritoneal irritation usually subsided in about 3 hours. No appreciable temperature elevation occurred during the time the animals were studied. Extensive granulomatous lesions resulted in all cases but no attempt was made to correlate the extent with serum polysaccharide levels. Average results of studies using 4 dogs are depicted in Fig. 1. The serum polysaccharide reached a maximal elevation on the third or fourth day and was higher than normal even on the 20th day. Intraperitoneal injection of 100 ml of sterile water in a control experiment caused only a slight change in the serum polysaccharide as noted in Fig. 1.

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Experimental Surgical Operation. Several experimental laparotomies were carried out under aseptic conditions on mongrel dogs, and the serum polysaccharide level was studied by serial determinations following operation. Since no tissue was removed during these procedures, the only tissue injury was that resulting from the incisions. Data obtained from these studies based on the results from 3 animals are presented graphically in Fig. 1. The maximal elevation of serum polysaccharide occurred on the fourth day. The elevation was not as great as that caused by severe bacterial infection or turpentine abscess. No significant temperature elevation was found.

Discussion. It is apparent from the data presented that an increase in serum polysaccharide follows the production of experimental inflammation regardless of the causative agent. As this response is a delayed one, with maximal elevation in 3 to 6 days after injury, the polysaccharide level does not appear to be directly correlated with tissue destruction. Furthermore, the same effect is produced by inflammatory processes caused by injection of talc suspensions or by intrapleural injection of turpentine, conditions in which little tissue destruction is thought to occur. Fever is apparently not essential for the elevation, inasmuch as the serum polysaccharide rose in cases where no temperature elevation occurred. It is therefore possible that the elevation of polysaccharide may be correlated with tissue proliferation or repair.

The length of time which elapsed before the polysaccharide level became normal again seemed to have some connection with the extent of repair. The time required for the polysaccharide level to become normal in an animal with a large abscess resulting from bacterial action was much longer than for types of inflammation where less regeneration of tissue occurred. It seems unlikely that the continued high level could be the direct result of tissue injury, as signs of infection and trauma were absent after the eighth day.

Summary and conclusions. Studies were made of serum polysaccharide levels in dogs following the production of sterile turpentine abscesses, of bacterial abscesses, and of talc granulomas, the injection of turpentine intrapleurally, and experimental surgical operations. In all cases the polysaccharide level rose and the maximal level depended somewhat upon the type of inflammation. Elevation occurred both in the presence and absence of fever. The maximal elevation appeared within 3 to 6 days after the initial injury. The suggestion is made that this phenomenon is correlated in some way with tissue proliferation or repair.

Received July 25, 1949. P.S.E.B.M., 1949, **72**.

Hydrolysis of Conjugated Steroids by Bacterial Glucuronidase. (17413)

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The use of acid for liberating the steroids of urine is based on the observation that these compounds are excreted in conjugated form as evidenced by their insolubility in fat solvents and by the isolation of steroid glucuronides[1-3] and sulfates.[4-6] The evidence that such treatment causes considerable loss[7-10] has led to a consideration of enzymes for the liberation of steroids from their conjugates.

Cohen and Marrian[11] and Patterson[12] have previously reported the liberation of estrogen of pregnancy urine by bacterial action and Barker, Brooksbank, and Haslewood[13] have found that pregnanediol glucuronide, and in some experiments glucuronic acid, which presumably was liberated from the conjugate were destroyed by some strains of *Staphylococcus albus*. Preparations possessing glucuronidase activity which were obtained from mammalian tissues hydrolyze the glucuronides of estriol,[14,15] pregnanediol[7,16] and other compounds[14,17,18] and Cohen and Bates[19] have studied the hydrolysis of the sulfuric acid esters of estrogens by an enzyme, phenolsulfatase, obtained from a mold, *Aspergillus oryzae*.*

Recently, we have reported[20] the production of glucuronidase by *E. coli* cultured in the presence of menthol glucuronide and an adequate oxygen supply. By this means potent culture filtrates were obtained which may be used without further purification or concentration. The present report deals primarily with the effect of this bacterial glucuronidase on the hydrolysis of some of the urinary steroids.

Experimental Procedures. The urines, preserved with thymol, were boiled at neutral pH for 10 minutes. Single specimens were boiled immediately after voiding and 24-hour samples at the end of the collection period during which time they were kept at 5°C. This precaution was taken because of the reports that glucuronidase[21] and phenolsulfatase[22] occur in urine. Free steroids were determined in these urines and the amounts of

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* Studies on hydrolysis of combined estrogens by phenolsulfatase which were discussed at the Federation meeting[20] in Detroit, April 1949, will be presented in detail in a subsequent report.

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TABLE I.
Comparison of the Influence of Different Hydrolytic Agents on Total Estrogen Liberated from Human Pregnancy Urine.*

	Method of hydrolysis					
	Total estrogen (Kober) as mg estrone per l				Total estrogen as mouse units of estriol per l	
Urine	HCl	Glucuronidase	None	Urine	HCl ×1000	Glucuronidase ×1000
1	5.38	4.88	.03	A†	109	147
2	16.27	15.00	.21	B†	155	207
3	10.5	12.3	.24	C†	120	213
4	13.87	17.25	.285	D†	320	460
5	6.75	9.75	.15	E†	176	266
6	12.0	13.3	.06			

* Obtained during the last month of pregnancy.

† Ether extracts of urines A-E were not fractionated into the phenolic and neutral fractions before assay. In the bioassay (Marrian), 20 animals per dose were used and estriol was used for the standard.[2]

estrogen and 17-ketosteroid liberated from their conjugates with glucuronidase compared with the amounts liberated by acid hydrolysis.

Acid hydrolysis was performed by refluxing the urine for 10 minutes with 15 vol. % concentrated HCl. For enzymic hydrolysis, the urines with added glucuronidase were incubated for 24 hours at 38°C at pH 6.5. All hydrolyzed and unhydrolyzed samples were adjusted to pH 3 and immediately extracted with ether. Phenolic and neutral fractions were separated according to Friedgood, *et al.*,[23] ketosteroids determined by the method of Holtorf and Koch[24] after removing the non-ketonic fraction[25] and total estrogens by Salter's[26] modification of the Kober method.

The extracts obtained from the glucuronidase hydrolyzed urine gave very much less background color with the Kober reagent (absorption at 420 mμ) and a more typical color with Zimmermann's reagent than that obtained after acid hydrolysis. Furthermore, a larger proportion of the color with m-dinitrobenzene is due to ketonic compounds reacting with Girard reagent T.

Under our experimental conditions, glucuronidase qauntitatively splits glucuronic acid from menthol glucuronide as measured by Fishman's modification[27] of Miller and Van Slyke's method,[28] and hydrolyzes 95% of estriol glucuronide. Furthermore, glucuronidase preparations tested with estrone sulfate and dehydroisoandrosterone sulfate contained neither phenolic nor alcoholic sulfatase.

Results. The data in Table I show that except for urines 1 and 2, higher values for total estrogen were obtained after hydrolysis with glucuronidase than with hydrochloric acid. In addition, the estimation is more accurate because of the lower background color after treatment with Kober's reagent. Although the lower values after acid hydrolysis may be due to incomplete hydrolysis as well as destruction, it is surprising that so large an amount of estrogen is conjugated with glucuronic acid.

With the urine of pregnant women as well as that of normal men and one case of pseudohermaphroditism, glucuronidase did not liberate as much ketosteroid as that liberated by hydrochloric acid (Table II). Undoubtedly

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TABLE II.
Comparison of Hydrochloric Acid and Glucuronidase Hydrolysis of the Ketosteroids of Human Urine.

Urine No.	Method of hydrolysis		
	HCl	Glucuronidase	No hydrolysis
	Normal men—mg* per 24 hr		
1	—	4.25	.488†
2	—	8.3	1.176†
3	—	7.2	.612†
4	—	4.05	.450†
5	—	4.75	.546†
6	14.4	6.7	.60†
7	11.0	5.1	.416†
8‡	26.2	14.0	.448†
	Pregnant women—mg* per l		
1§	0.92	1.2	.07
2	5.5	5.0	.18
3	5.8	5.0	.26
4	10.8	7.4	.42
5	6.3	5.6	.21
6	4.8	3.6	.16

* Expressed as dehydroisoandrosterone.
† Micro-Girard separation not made.
‡ Pseudohermaphrodite.
§ Pregnancy urines 1-6 are the same as those recorded in Table I.

ketosteroid conjugated with sulfuric acid accounts for at least a part of this difference but here again, it is surprising to find so large a proportion of the ketosteroid fraction in combination with glucuronic acid.

The quantities of free estrogens and ketosteroids recorded in Tables I and II are of interest since they were obtained from fresh urine in which enzymes, if present, had been destroyed by boiling. However, even these low values may be due to enzymic hydrolysis within the bladder.

As would be expected, bacterial glucuronidase hydrolyzed sodium pregnanediol glucuronidate resulting in a 90% recovery of pregnanediol as determined by the method described by Talbot, *et al.*[29]

Dr. Ralph A. Kinsella, Jr. of this department, in preliminary studies has found that glucuronidase markedly increases the amount of cortical steroids of the urine of normal adult men as determined by a somewhat modified method of Heard, *et al.*[30]

Summary. Bacterial glucuronidase which hydrolyzes the glucuronides of estriol, pregnanediol, menthol and phenolphthalein liberates a large proportion of estrogen, ketosteroid and corticosteroid of human urine.

We are deeply indebted to Professor G. F. Marrian for estriol glucuronide; to Dr. A. Stanley Cook of Ayerst, McKenna and Harrison for estrone sulfate; to Dr. H. L. Mason for dehydroisoandrosterone sulfate; to Dr. Willard Allen for pregnanediol glucuronide, and to Miss Corinne Dewes, Robert A. Doisy, and Joseph Yglesias for invaluable laboratory assistance.

Received August 1, 1949. P.S.E.B.M., 1949, **72**.

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Prostigmin on the Ovaries and Uteri of Albino Rats.* (17414)

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Prostigmin is a parasympathomimetic drug that exerts some of its effects through its ability to inhibit cholinesterase and enhance the action of acetylcholine. Soskin, Wachtel, and Hechter[1] showed that prostigmin will start the menstrual flow in the non-pregnant, but would have no effect in the pregnant human. On this basis they proposed its use as a pregnancy test. This idea had its beginning in 1939 when Reynolds[2] reported that estrogens caused a transient increase in the amount of acetycholine in the uterus of rats, and that the uterus thereby becomes hyperemic. Since that time several authors[3-9] have reported using prostigmin as a valuable test for pregnancy and treatment of 'nervous' amenorrhea in the absence of organic dysfunction. In a subsequent paper Taubenhaus and Soskin[10] attempted to explain this effect of prostigmin from a study of the hypothalamic region and the release of luteinizing hormone. They offered evidence that prostigmin applied directly to the pituitary will cause a release of LH and a transient corpus luteum formation.

Since this drug is now commonly used it seemed desirable to know more about its action on the reproductive system of the female. The present report deals with such a study in albino rats.

Procedure and results. Female rats of the Wistar strain were used. Prostigmin was injected twice daily. The daily dose for all small rats was .04 mg and for mature females 0.1 mg given in physiological salt solution in dilution of 0.1 mg of prostigmin per cc. Five groups of young female rats are shown in Table I. Group 1 consisted of a group of immature virgin females which weighed approximately 45 g at the start. The control animals received 1.5 units of gonadogen† daily, and the test animals also received the same amount of gonadogen plus 0.04 mg of prostigmin‡ daily. Gonadogen was given in order that the immature uterus would be in a hyperemic state at the time prostigmin was given and would, therefore, be more like the premenstrual endometrium of the human than would the uterus of the untreated immature rat. After 5 days of treatment, the animals were sacrificed, the uteri and ovaries were weighed and desiccated for a period of 8 days, 4 days at 80°C followed by 4 days in a desiccator containing calcium chloride. The weights of these fresh organs are shown in Table I. Both ovaries and uteri are heavier in the prostigmin injected rats. Statistical analyses, using the mean difference divided by the standard error of the difference, are shown under "significant ratio" (S.R.) Any figure in this column (Table I) should be at least 3 in order to say that a significant change in weight has occurred between two means. In the present study ovaries and uteri of the prostigmin injected rats are compared with controls. In Group I the "significant ratio" was 2 for the ovaries and the same

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* Research paper, Journal Series No. 891, University of Arkansas.

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† Gonadogen was kindly supplied by the Upjohn Company.

‡ We are indebted to Hoffman-La Roche for the Prostigmin.

TABLE I.

Procedures				Ovaries			Uteri		
Group	No. of rats	Kind of inj.	Autopsy body wt, g	Wt in mg	S.R.	Odds	Wt in mg	S.R.	Odds
				Immature females					
1	20	G.	63	139	2.0	2-1	136	2.0	20-1
	20	G. & P.	57	156			151		
2	10	S.	81	23	1.2	7-1	68	2	10-1
	10	P.	81	19			55		
				Immature females: ovariectomized					
3	21	St.	103	—			250	3.5	120-1
	21	St. & P.	102	—			272		
				Mature females					
4	16	S.	147	51	1.0	4-1	186	3.0	60-1
	16	P.	140	53			202		
5	19	S.	206	70	2.7	100-1	410	1.5	4-1
	19	P.	198	81			449		

The wet wt of the ovaries and uteri are shown. G = Gonadogen, P = Prostigmin, S = Saline controls, and St = Stilbestrol.

S.R.—Significant ratio which is based on S.D. (stand. dev.) = $\sqrt{\frac{\Sigma d^2 f}{n}}$; S.E.—(standard error) = $\frac{S.D.}{\sqrt{n}}$ and standard error of the difference = $\sqrt{(S E_1)^2 + (S E_2)^2}$.

for the uteri.

Some investigators[11,12] believe that in cases of paired data the method of Student[11] should be used. This method uses the standard deviation and by tables[11,12] the odds that such results will occur again can be determined. We have analyzed the data by this method as shown in Table I. It will be seen that the odds for Group 1 are 2-1 for the ovaries and 20-1 for the uteri. In contrast to this, Group 2 shows that prostigmin alone did not enlarge the uterus of the immature rat, in fact the mean weights of ovaries and uteri were somewhat smaller after prostigmin as compared with saline (0.9%) controls (Group 2 Table I). In other words the uterus must be in a congested state before the effects of prostigmin can be detected.

Group 3 consisted of ovariectomized virgin female rats in which the controls received 0.25 mg stilbesterol daily while the test animals received 0.25 mg of stilbesterol plus 0.05 mg prostigmin daily. Treatment was begun 4 or 5 days after operation and was continued for 7 days. The animals were then similarly sacrificed and the uteri weighed and disiccated. This group was designed to test prostigmin on the hyperemic uterus in the absence of ovaries. It will be noted that the increase in weight of these uteri was at least as much as that found in Group 1 where the ovaries were present. The figures of 250 and 272 for the weights on the uteri give a difference of 8.8%. The "significant ratio" of 3.5 shows that this weight difference is significant and when analyzed by Student's method the odds are 120 to 1, which are the largest in Table I.

These experiments with immature females lead us to consideration of prostigmin studies in sexually mature female rats. These data are shown in Groups 4 and 5 (Table I). The only difference between these two groups is the duration of injection which was 7 and 30 days in Groups 4 and 5 respectively. Both groups received 0.1 mg of prostigmin daily.

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[12] Love, H. H., *J. A. Soc. Agronomy*, 1924, **16**, 70.

Although the ovarian weights were unchanged in the seven day group and only slightly larger in those injected thirty days there was an increase in the percentage weight of the uteri in both Groups (4 and 5, Table 1) which showed an analysis to have a "significant ratio" of 3 and 1.5 in Groups 4 and 5 respectively (Table I). Since uterine weights are more variable than those of the ovaries these "significant ratios" for the uteri are all the more interesting.

Pathology-histological examination§ showed no consistent change in the ovaries. The uteri of the castrated prostigmin rats (Group 3) show the most changes; there were more round cells, edema and inflammatory cells, both polynuclear leucocytes and lymphocytes, than in the controls. The changes were almost completely confined to the submucosa. In the castrated controls both polynuclear leucocytes and lymphocytes are present but not numerous. Some edema and inflammatory cells were found in the submucosa cell groups including the controls. No changes in epithelial or musculature structure were observed in the prostigmin injected rats. The difference between the prostigmin and control animals is minor and pathologically there is no marked alteration.

Discussion. The ovaries of prostigmin injected rats were apparently in good condition as seen histologically and by the estrous cycles. Twenty additional rats injected twice daily with 0.5 mg of prostigmin at each injection showed no significant change in estrous cycles as compared to saline injected controls. We were impressed by the fact that when the background was a mature uterus, induced by age as in Groups 4 and 5, or in hyperemic state brought about by estrogens Groups 1 and 3, prostigmin further increased the weight. This is also evident after corrections are made for differences in body weight and after desiccation. Thus in Group 3 the uteri in the prostigmin injected rats were 8.8, 9.9 and 9.2 percent heavier in the uncorrected, corrected for body weights and desiccated tissues respectively. This clearly shows that the increase in weight of the uterus after prostigmin was not due to edema.

Summary. Prostigmin was given to young rats in daily amounts of 0.04 to 0.1 mg. The ovaries were slightly increased in weight. The uteri if mature as the result of age or estrogens showed increase in weight following injections of prostigmin and these changes were present after desiccation. Estrous cycles showed no significant change from normal. Cellular elements of inflammatory cells were more abundant in the submucosa after prostigmin. On the whole, pathological changes were not marked; therefore, from the pathological viewpoint effects of prostigmin are minor as far as changes in the ovaries and uterus are concerned.

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§ Histological and pathological studies were kindly made by Doctor A. Nettleship of the Pathology Department.

Received August 1, 1949. P.S.E.B.M., 1949, **72**.

Essential Hypertension. Therapeutic Trial of Veriloid, a New Extract of *Veratrum viride*.* (17415)

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Veriloid (Coe Chemical Company), a purified alkaloidal fraction of *Veratrum viride*,[1]

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* Supported in part by Grants-in-Aid from the Coe Chemical Company, and the Squibb Institute for Medical Research.

† Present address: Georgetown University Medical Center, Washington, D.C.

[1] Stutzman, J. W., Maison, G. L., and Kusserow, G. W., PROC. SOC. EXP. BIOL. AND MED., 1949, **71**, 725.

has been found to be an active hypotensive agent in hypertensive patients. Effective by mouth, it has produced fewer toxic side effects (nausea and vomiting), than any other active oral preparation presently available. This preliminary report summarizes our experience to date with this agent.

Methods and Results. The subjects were clinic and private cases of established essential hypertension. In 10 hospitalized patients the drug was given acutely after a control period of at least 48 hours of bed rest. Measurements of arterial pressure and pulse rate were made by 2 independent observers at least 3 times a day, and (when these had stabilized) every half hour for the 2 hours before and 4 hours after the acute administration of the drug. The average (to the nearest 5 mm Hg) of the 4 measurements immediately before, and of the 4 lowest consecutive measurements after the administration of the drug were used to characterize its action.‡ In the 10 patients the average arterial pressures were lowered as follows: 220/125 to 155/90, 220/130 to 135/95, 220/140 to 135/90, 180/100 to 150/90, 190/125 to 160/110, 190/125 to 180/115, 195/100 to 170/95, 230/140 to 200/115, 190/125 to 165/110, and 240/130 to 190/110 mm Hg. In only 3 patients was there nausea, and in only 1 of these vomiting.

In 25 ambulatory cases at least 3 measurements were made of arterial pressure and pulse rate under standard conditions (sitting and/or lying) during each visit. After suitable control observations the patient was instructed initially to take 1, or (in large persons) at most 2, mg of Veriloid 4 times a day at intervals of at least 4 hours (after each meal and at bedtime§), unless this caused nausea, in which case he was to reduce the next dose, or if still nauseated to omit it entirely. After 1 week he was to return for a check-up, at which time, unless satisfactory results had already been achieved, he was instructed gradually to increase the dosage by not more than 1 mg a day (provided this caused no symptoms), adding 0.5 to 1 mg first to the bedtime, then to the after-breakfast, the after-lunch, and the after-supper doses, in that order, and to return for weekly checkups.

In this way, patients were gradually worked up to their nauseating dose, and then maintained at a slightly reduced dosage which averaged 2 mg of Veriloid 4 times a day at the end of 3 to 4 weeks. Patients were repeatedly cautioned, if nauseated or otherwise uncomfortable, to remain recumbent and to reduce slightly their subsequent doses. On this regimen, severe collapse reactions were rare after Veriloid. That definite toxic reactions could occur had been discovered during acute trial of the drug given in single doses of 4 to 6 mg. The antidote used for these reactions was the same as for those after other Veratrum preparations, namely, ephedrine sulphate intramuscularly (30 to 45 mg) and/or atropine (0.5 to 1 mg). Similar, but milder reactions occasionally occurred in patients on continued administration. Characteristically, these appeared only after several weeks of treatment and required a further small reduction of dosage. However, in only 3 patients was it necessary to discontinue the drug altogether.

Of 25 patients maintained to date on 6 to 11 (average 8) mg of Veriloid a day, 9 were followed for 12 to 20 weeks and had their average arterial pressures lowered as follows: 245/135 to 190/100, 250/150 to 210/110, 235/135 to 185/115, 235/150 to 230/135, 205/110 to 180/90, 180/120 to 140/85, 175/110 to 160/90, 180/115 to 150/95, and 190/110 to 155/90 mm Hg. Eleven patients took the drug for 6 to 12 weeks and had their average pressures changed from: 200/130 to 150/90, 160/100 to 130/80, 210/130 to 140/105, 250/140 to 170/80, 230/135 to 185/110, 210/140 to 150/90, 235/115 to 195/105, 185/110 to 170/95, 210/110 to 195/100, 195/110 to 180/110, and 185/90 to 175/80 mm Hg. Five patients were followed for 2 to 4 weeks and had pressures changed as follows: 260/150 to 190/110, 180/110 to 140/90, 200/115 to 180/110, 190/115 to 170/100, and 200/115 to 170/100 mm Hg.

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‡ The method of acute administration of the drug will be reported in detail later.[2]

[2] Wilkins, R. W., Stanton, J. R., and Freis, E. D., to be published.

§ In animals[1] as well as in man, *Veratrum viride* is less nauseating when food is already in the stomach and/or the subject is lying down. However, if food is taken within 3 hours after the drug, nausea is liable to occur.

Thus, on chronic as well as acute administration Veriloid was an active hypotensive agent, clinically similar to other Veratrum preparations previously tested.[3-5] Relative to its hypotensive effects, however, it appeared to produce somewhat less nausea and vomiting, the chief symptoms found in previous experience to interfere with the long-term usage of *Veratrum viride*. As with other preparations, meticulous adjustment of dosage to patient was the most important single point in the chronic use of Veriloid. Patients objected so strongly to the symptoms associated with overdosage that it was the practice in the present study to be satisfied with moderate lowerings of arterial pressure, rather than to attempt dramatic effects at the expense of even occasional unpleasant side reactions.

Summary. In hypertensive patients Veriloid is an active hypotensive agent when administered acutely, or chronically for periods as long as 5 months. Meticulous regulation of dosage is important in obtaining optimum results. After several weeks of continuous therapy it is frequently necessary to reduce the dosage in order to avoid nausea and vomiting. Nevertheless, relative to its hypotensive effects Veriloid seems to produce fewer toxic reactions than any other oral preparation of *Veratrum viride* presently available.

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[3] Freis, E. D., and Stanton, J. R., *Am. Heart J.*, 1948, **36**, 723.

[4] Freis, E. D., Stanton, J. R., Culbertson, J. W., Litter, J., Halperin, M. H., Burnett, C. H., and Wilkins, R. W., *J. Clin. Invest.*, 1949, **28**, 353.

[5] Wilkins, R. W., Freis, E. D., and Stanton, J. R., *J.A.M.A.*, 1949, **140**, 261.

Received August 3, 1949. P.S.E.B.M., 1949, **72**.

Oral Toxicity of a New Miticide, Di-(p-chlorophenyl)methylcarbinol.* (17416)

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Di-(p-chlorophenyl)methylcarbinol(DMC) synthesized by Bergmann and Bondi,[1] and later by Grummitt *et al.*,[2] has been shown to possess marked miticidal properties.[3] A preliminary study of its acute and subacute toxicity for rats and mice, following oral administration, is the subject of this report.

Acute Toxicity. Young albino rats (80-100 g) and adult albino mice (18-22 g), of the female sex, were given single doses of DMC by stomach tube. Solutions in corn oil and suspensions in aqueous mucilage of tragacanth (2%) were used. Mice were observed for 10 days, and rats for 21 days, following intubation. The results of these experiments are shown in Table I.

With the oil solution of DMC the largest single dose tolerated by all 5 rats in a dosage group was 250 mg/kg. With the aqueous suspension one animal died following a dose of 375 mg/kg, although no fatalities resulted from dosage with 500 mg/kg. Fatalities from larger doses usually occurred 48 to 96 hours after administration. Urinary incontinence, tremors, ataxia, and impairment of righting reflexes were present for approximately 24 hours before death. Such abnormalities were not seen in animals which survived. Autopsies performed immediately after death revealed no gross pathological changes beyond

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* This study was supported by a grant-in-aid from the Sherwin-Williams Co., Cleveland, Ohio.

[1] Bergmann, E., and Bondi, A., *Berichte*, 1931, 64B, 1455.

[2] Grummitt, O. J., Buck, A., and Becker, E., *J. Am. Chem. Soc.*, 1945, **67**, 2265.

[3] Witman, E. D., and Waters, H. A., unpublished observations.

TABLE I.
Acute Toxicity of Di-(p-chlorophenyl)methylcarbinol for Rats and Mice Following Oral Administration by Stomach Tube.

	Rats		Mice			
	Oil solution	Aq. suspension	Oil solution		Aqueous suspension	
Dose, mg/kg	Mortality ratio		Paralysis ratio	Mortality ratio	Paralysis ratio	Mortality ratio
100			0/5	0/5	0/5	0/5
175			0/5	0/5	0/5	0/5
250	0/5	0/5	0/5	0/5	2/5	0/5
375	2/5	1/5	1/5	0/5	1/5	0/5
500	2/5	0/5	1/5	0/5	2/5	0/5
750	2/5	3/5	4/5	1/5	4/5	1/5
1000	2/2	4/9	4/5	1/5	5/5	3/5

TABLE II.
Effect of a Diet Containing DMC on Weight and Ultimate Survival of Young Albino Rats.

	Avg wt in g (10 rats per group) Time in days								
% DMC in diet	0	5	10	19	26	35	42	63	70
.0	63.6	80.0	84.6	112	140	171	194	248 SE = 8.3	262 SE = 8.4
.1	63.6	73.7	85.8	113	140	176	194	226 SE = 6.5	245 SE = 7.8
.25	63.8	70.2	77.4	102(7)[*]	127(7)	172(4)[†]	177(4)	206(4)	220(4)
1.0	63.7	All dead after 72 hr							

[*] 3 animals dead between 10th and 19th days.
[†] 3 animals sacrificed 28th day.

a moderate degree of hyperemia of the peritoneum and the abdominal viscera.

Mice survived the oral administration of 500 mg of DMC per kg, both as an oil solution and as an aqueous suspension, while dosages of 750 or more mg per kg resulted in the death of one or more of the 5 animals in the dosage group. During a period of 12 to 72 hours prior to death, mild to severe motor impairment of the posterior extremities was seen. The front limbs also were involved in a few cases. The motor signs rarely developed in less than 12 hours after administration of DMC. Recovery, rather than death, followed in some cases, a situation in sharp contrast to that seen in rats.

Subacute Toxicity. Four groups of 10 young female albino rats were fed, *ad libitum,* a powdered diet[†] containing 1.0, 0.25, 0.1, and 0.0% of DMC, respectively. The individually-housed animals were inspected daily, and were weighed periodically, but no attempt was made to measure food consumption. The progress of these animals is recorded in Table II.

The animals receiving the highest level of DMC (1.0%) became anorexic after 24 hours. Mild depression characterized their subsequent behavior and death occurred within 72 hours. The disturbances of locomotion and equilibrium seen after large single doses of DMC were not observed here. Autopsies performed immediately after death revealed hyperemia of the abdominal viscera as the sole gross finding.

The rats given the intermediate level of DMC (0.25%) consumed noticeably less food than the controls during the first 5 days. The intake of 7 of these animals increased and grossly equalled that of the controls during the next 5 days. The remaining 3 instead became mildly depressed and completely anorexic. They died between the tenth and nineteenth experimental days. The survivors remained normal in appearance and behavior. Three were sacrificed on the twenty-eighth day and the remaining 4 at the end of the experiment. Mild hyperemia of the peritoneum and abdominal viscera was the only gross finding at autopsy in all 10 animals. There was no gross difference in average body weight between the few survivors and the controls.

[†] General Mills "Checkers."

The third group of rats whose diet contained 0.1% DMC showed a slight, though not significant, weight lag on comparison with the control group at 5 days. Between this time and the ninth week the average weights of the two groups were almost equal. Statistical analysis of the difference of 22 g between the average weights at 9 weeks yielded a critical ratio[‡] of 2.2 which borders on significance. One week later the weight difference was 17 g and the ratio was only 1.5. Since the experiment was terminated at this time it is impossible to say whether these differences heralded the beginning of a significant lag in weight gain, and other signs of toxicity, in the treated group. The appearance and behavior of the two groups of rats did not differ in any way. Autopsies performed when the animals were sacrificed revealed no gross evidence of pathological changes in any organs or tissues.

Comment. These studies reveal that the toxicity of DMC is of the same order as that demonstrated by Woodard *et al.*[4] for DDT under grossly similar conditions. However, a more extensive comparison of the two compounds in the same laboratory would be necessary to determine accurately the relative toxicity of the two compounds.

Summary. 1. Rats and mice were intubated with single doses of a new miticidal compound, di-(p-chlorophenyl)methylcarbinol (DMC). The effect of graded doses on ultimate survival is discussed and the ability of large doses to produce signs suggestive of nervous impairment is described.

2. A powdered diet containing 0.1% DMC was well tolerated by growing rats during a ten week period.

3. The results compare favorably with those obtained in a similar limited study of DDT in another laboratory.

[‡] $\dfrac{M_1 - M_2}{\sqrt{SE^2_{M_1} + SE^2_{M_2}}}$

[4] Woodard, G., Nelson, A. A., and Calvery, H. O., *J. Pharm. Exp. Therap.*, 1944, **82**, 152.

Received August 5, 1949. P.S.E.B.M., 1949, **72**.

Effects of Inunction of Alpha-Estradiol on Testes and Thyroids of Albino Rats.* (17417)

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A diverse opinion exists in regard to the effect that the administration of sex hormones has on thyroid activity. Many investigators have noted that the thyroid remains normal or shows a slight degree of hypo-activity after castration or the administration of female sex hormones.[1–10] In contrast many workers have noted that the administration of female sex hormones cause a hyperactivity of the thyroid.[11-17] Investigations at present show that estrogenic substances exert an inhibitory influence on the structure and size of the testes and their accessories.[5,18-25] Some workers have obtained opposite results.[10,26]

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* This work was carried out at the Department of Anatomy and Physiology, at the University of Kentucky, Lexington, under the direction of Professor R. S. Allen.

[1] Chouke, S., *Endocrinology*, 1930, **14**, 12.

[2] Aron, M., and Benoit, J., *Compt. rend. de Soc. Biol.*, 1932, **109**, 923.

[3] Starr, P., and Bruner, R. C., PROC. SOC. EXP. BIOL. AND MED., 1935, **33**, 465.

[4] Shumacker, H. B., and Lamont, A., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 568.

[5] Uotila, U. U., *Endocrinology*, 1940, **26**, 123.

[6] Zalesky, M., *Anat. Rec.*, 1935, **62**, 109.

The present investigation was undertaken because of the conflicting results that have been obtained.

Materials and methods. Three groups of male albino rats were used. Eight male rats of Group 1 (40 days of age) were castrated 2 weeks after the descent of the testes into the scrotum. Treatment began the day following castration and consisted of daily inunction of 270 rat units[†] of alpha-estradiol[‡] in ointment form to the skin surface of the interscapular region. Group 2 consisted of eight, 15 day old normal non-castrated immature male rats. Treatment was carried out as in Group 1 but was started before the descent of the testes into the scrotum. Six rats in Group 3 served as control animals and received no treatment. On the day following the 24th application of alpha-estradiol on the experimental rats all the animals were killed with chloroform. The thyroids and testes were fixed in Bouin's fluid and the sections were stained with Ehrlich's acid hematoxylin and eosin. Klein's method[27] was employed for studying the size of the thyroid follicles and seminiferous tubules. The height of the follicular epithelium was determined by the method of Rawson and Starr.[28] An estimated percentage of destruction of the follicular walls was made by comparison with the findings of Morrell and Hart[25] who determined in percentage, the area of a section showing destruction. One hundred follicles from a section of the thyroid of each animal were studied for the number of follicles containing wall vacuoles in the colloid as described by Möllendorff.[29] The average number of primary and secondary spermatocytes was taken from 20 typical tubules in a section from each animal. The testes were compared as to gross size and as to the interstitial elements of Groups 2 and 3.

Observations. Thyroid follicular size and epithelial height were used to determine an increase or a decrease in thyroid activity. The results are shown in Table I. The long diameter of the thyroid follicles of Groups 1 and 2 averaged respectively 113.8 and 96.1 μ, while in the normal rats (Group 3) it was noticeably less with an average of 66.9 micra. The height of the follicular cells in Groups 1 and 2 averaged respectively 15.3 and 15.6 μ, while group 3 averaged 34.5 μ. Due to the

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[7] Costantini, P., *Rass. di Clin. Ter. e Sc. Aff.*, 1929, **10**, 718.

[8] Sherwood, T. C., *Endocrinology*, 1940, **26**, 693.

[9] Sherwood, T. C., *Endocrinology*, 1941, **29**, 215.

[10] Korenchevsky, V., and Dennison, M., *J. Path. and Bact.*, 1935, **12**, 323.

[11] Kunde, M. M., *et al.*, PROC. SOC. EXP. BIOL. AND MED., 1930, **28**, 122.

[12] Benazzi, M., *Boll. Soc. Ital. Biol. sper.*, 1933, **8**, 790.

[13] Leiby, G. M., PROC. SOC. EXP. BIOL. AND MED., 1933, **31**, 15.

[14] Pincus, G., and Werthessen, N., *Am. J. Physiol.*, 1933, **103**, 631.

[15] Amilibis, E., *et al.*, *Klin. Wochen.*, 1936, **15**, 1001.

[16] Nathanson, I. R., *et al.*, PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 737.

[17] Alexin, M., *Arch. f. Gyn.*, 1940, **159**, 432.

[18] Golding, G. L., and Ramirez, F. T., *Endocrinology*, 1939, **12**, 804.

[19] Burrows, H., *J. Path. and Bact.*, 1936, **42**, 161.

[20] Greep, R. C., *et al.*, *Anat. Rec.*, 1936, **65**, 261.

[21] Gardner, W. U., *Anat. Rec.*, 1937, **68**, 339.

[22] Ringoen, A. R., *Anat. Rec.*, 1938, **72**, 118.

[23] Pfeiffer, C. A., and Gardner, W. U., *Anat. Rec.*, 1938, **72**, 59.

[24] van Wagenen, G., *Anat. Rec.*, 1935, **73**, 387.

[25] Morrell, J. A., and Hart, G. W., *Endocrinology*, 1941, **19**, 995.

[26] Allanson, M. J., *Exp. Biol.*, 1931, **8**, 389.

[†] The author wishes to thank Dr. W. H. Stoner of the Schering Corporation at Bloomfield, N. J., for supplying the alpha-estradiol (Progynon D-H) used in this experiment.

[‡] Each application consisted of 0.15 g of ointment which contained 0.0225 mg of crystalline estradiol having a rat unit value of 270.

[27] Klein, J., *Ann. Int. Med.*, 1935, **8**, 798.

[28] Rawson, R. W., and Starr, P., *Arch. Int. Med.*, 1938, **61**, 726.

[29] Möllendorff, W., *Handbuch Mikroskop. Anat. des Mensch*, 1939, **6**, 61.

TABLE I.
Histological Changes Occurring in the Thyroid of Groups I and II as Compared to Group III, Which Was Considered Normal.

Group		Long diameter of follicles, μ	Height of follicular epithelium, μ	Vacuolated colloid per 100 follicles (wall vacuoles)	Estimated % of follicular damage
I Castrated and treated	1	90.7	16.9	16	25
	2	108.7	17.2	31	25
	3	113.9	16.7	28	40
	4	120.5	13.6	35	40
	5	103.1	15.4	15	25
	6	116.4	15.7	43	40
	7	134.6	14.1	23	10
	8	122.6	13.7	39	10
	Avg	113.8	15.3	28.7	26.8
II Non-castrated and treated	1	99.5	17.8	26	0
	2	94.1	17.8	20	0
	3	83.0	13.2	23	10
	4	101.3	15.1	15	10
	5	95.4	13.7	31	25
	6	89.4	13.5	23	25
	7	110.0	21.2	19	10
	8	96.2	13.0	16	10
	Avg	96.1	15.6	21.6	11.2
III Normal control	1	63.0	23.2	7	0
	2	64.5	25.3	6	0
	3	70.5	39.7	4	0
	4	67.1	27.7	2	0
	5	74.7	52.0	8	0
	6	62.0	39.1	3	0
	Avg	66.9	34.5	5	0

increased follicular size and the lower epithelium of Groups 1 and 2 as compared with the normal animals it is apparent that hypoactivity of the thyroid gland was produced by the inunction of alpha-estradiol. The marked difference between Groups 1 and 2 indicates a greater hypothyroid condition in Group 1. This might be explained by the presence of the testes in Group 2, causing a higher androgen concentration.

Because of the greater increase of wall vacuoles in the colloid of the thyroids in Groups 1 and 2 in contrast to the lesser number in Group 3, mention is made of them although no physiological significance is attached to them at present. The wall vacuoles according to Möllendorff are formed by the contraction of the colloid due to fixation. All thyroids of this series were fixed for the same length of time in Bouin's fluid.

Damage to the follicular walls was greater in the castrated and treated rats than in the non-castrated, treated ones and was lacking in the normal. Morrell and Hart[30] report that the thyroids of male and female rats treated with stilbesterol show destruction of the follicles brought about by invasion of leucocytes which may result from the static condition of the gland. The destruction occurred in areas which appeared hyalinized in some cases and fibrous in others. The damage to the thyroid follicles that appeared in Groups 2 and 3 was similar to that of Morrell and Hart. Fibrosis was noticeable in all the damaged thyroids and the hyalinized areas appeared to be due to release of follicular colloid in the tissue spaces.

Alpha-estradiol prevented the descent of the testes into the scrota of the animals of Group 2 (Table II). According to Moore and Price[31] this is due to the inhibitory effect of estrogens upon the hypophysis by reducing

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[30] Morrell, J. A., and Hart, G. W., *Endocrinology*, 1941, **29**, 769.

[31] Moore, C. R., and Price, D., PROC. SOC. EXP. BIOL. AND MED., 1930, **28**, 38.

TABLE II.
Histological Changes Occurring in the Testes of Group II as Compared to Group III, Which Was Considered Normal.

								Avg No. per 20 tubules				
		Location	Size of tubules (Long diam.) in μ	Size of testes compared to controls (Group III)	Sustentacular cells compared to controls (Group III)	Spermatogonia	Mitosis of spermatogonia	No. of primary spermatocytes	No. of secondary spermatocytes	Spermatids	Spermatozoa	Degeneration of tubular walls
Group II Non-castrated treated	1	*	116.4	½	‡	§	§	21.7	18.0	—	—	—
	2	*	130.1	½	‡	§	§	30.3	23.7	—	—	—
	3	*	121.9	½	‡	§	§	19.0	24.0	—	—	—
	4	*	136.9	½	‡	§	§	16.9	14.2	—	—	—
	5	*	116.4	½	‡	§	§	12.0	5.0	—	—	—
	6	*	112.3	½	‡	§	§	15.6	18.0	—	—	—
	7	*	149.0	½	‡	§	§	12.9	28.3	—	—	—
	8	*	147.9	½	‡	§	§	11.6	10.5	—	—	—
	Avg		128.8	½				17.5	17.7			
III Normal control	1	†	257.5		‡	§	§	58.6	142.4	§	§	—
	2	†	325.3		‡	§	§	73.1	169.3	§	§	—
	3	†	282.1		‡	§	§	69.0	154.2	§	§	—
	4	†	239.7		‡	§	§	87.6	193.4	§	§	—
	5	†	343.8		‡	§	§	78.4	181.4	§	§	—
	6	†	304.8		‡	§	§	62.0	149.3	§	§	—
	Avg		292.2					71.2	165.0			

* Abdomen. † Scrotum. ‡ Normal. § Present. — Absent.

its gonadotropic secretion. The testes of Group 2 did not remain in the abdomen long enough for all of the germ cells to disappear from the tubules. Spermatogenesis was reduced to the point that spermatid formation was completely absent. Probably these changes in the tubules were due to the alpha-estradiol indirectly, by preventing testicular descent, and permitting cellular changes due to retention in the body cavity. There was no noticeable change in the interstitial elements. The gross size of the testes were half that of the control animals.

Summary. Daily inunction for 24 days of 270 rat units of alpha-estradiol (Progynon D-H) to normal and castrated immature male rats produced noticeable changes in the thyroid glands and in the testes of the non-castrated males. The thyroid glands appeared, histologically, in a hypoactive state. They exhibited an increased colloid content and a low follicular epithelium and destruction to the follicles. The hypoactivity appeared greater in castrated rats than in the non-castrated. Descent of the testes in immature males was prevented by the alpha-estradiol.

Received August 8, 1949. P.S.E.B.M., 1949, **72**.

Effects of Injection of Large Molecular and Particulate Substances on Body-Temperature of Rats.* (17418)

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In an earlier paper[1] it was shown that autolyzed yeast and the magnesium salt of yeast nucleic acid produce a rise in the body-temperatures of albino rats when injected subcutaneously, but produce a fall in the body-temperatures when injected intraperitoneally. On the other hand, the purines, pyrimidines and allantoin derived from nucleic acid do not materially affect body-temperature by either route of administration. It seemed possible, therefore, that the property of altering body-temperatures may be associated with the high molecular weight or particulate nature of the substances employed rather than with chemical groups present. India ink injected subcutaneously, as well as autolyzed yeast, has been used for producing fever experimentally. India ink is also known to be phagocytized after intraperitoneal injection and taken up by the cells of the reticuloendothelial system where it is easily seen. However, we have not found any record of body-temperature measurements after intraperitoneal injections of this substance.

In this paper we are reporting experiments with India ink, with blood charcoal and with two dyes that have been used for visualizing the lymph channels and the reticuloendothelial system, carmine and trypan blue. In addition to these, a variety of substances was tested either because of their particulate nature or because they have been reported to alter body-temperature. In the latter group were skim milk, barium sulfate as prepared for gastrointestinal roentgenography, lipiodol, egg albumin, and 2, 4-dinitrophenol. Of these, only the last named showed any influence on body-temperature by either subcutaneous or intraperitoneal administration, and it produced fever by both routes. Because of the negative results no data are reported for this group of substances.

It seemed possible that the fall in body-temperature observed after intraperitoneal injection of some substances was a response to shock. To test this hypothesis blood cell volumes and plasma proteins were measured before and at intervals after treatment. These measurements are compared with similar measurements made after intraperitoneal injections of ox bile and solutions of peptone, two substances that are used for the experimental production of shock.

Materials and methods. The carmine used was a Grubler and Company preparation. The trypan blue was obtained from the Coleman and Bell Company. For most of the experiments both were prepared for injection by shaking 100 mg of the dye with 100 cc of 0.9% NaCl solution. Neither dye was completely soluble at this concentration, the carmine less so than the trypan blue, but both remained suspended long enough after vigorous shaking for injection. Except as indicated, Higgins India ink was used as purchased. Two g of Merck's blood charcoal were shaken vigorously with 100 cc of 0.9% NaCl solution just before injection. Ox bile was used as received from the packing house. The peptone was a 20% aqueous solution of J. T. Baker's powdered "Peptone, Bacteriological." Blood for cell volume and plasma protein determinations was obtained by clipping the tail and allowing the blood to drop into a small cuvette containing heparin. Cell volumes were determined with Smith tubes and plasma proteins by the copper sulfate specific gravity method.[2] The methods of

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* Supported under contract with the Office of Naval Research.

[1] Hill, R. M., and Rutledge, E. K., Proc. Soc. Exp. Biol. and Med., 1949, **71**, 9.

[2] Phillips, R. A., Van Slyke, D. D., Dole, V. P., Emerson, K., Hamilton, P. B., and Archibald, R. M., Publication of the United States Navy Research Unit at the Hospital of the Rockefeller Institute for Medical Research.

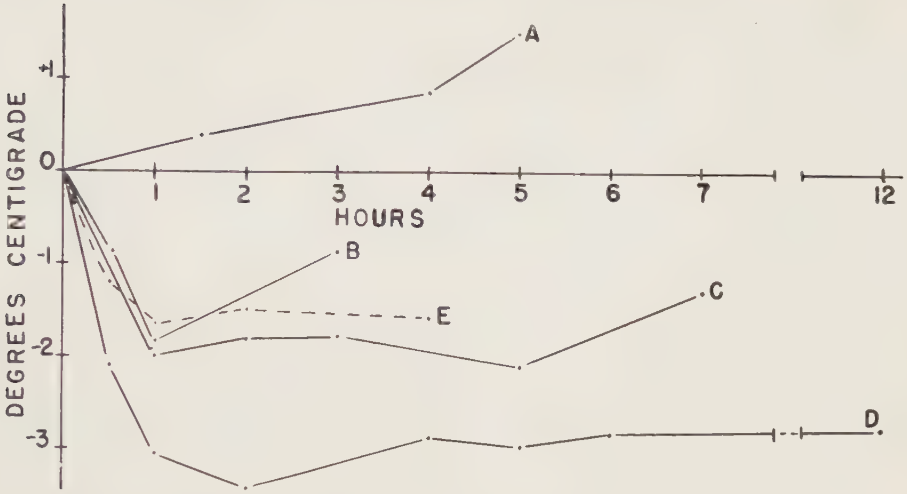

FIG. 1.

Average body-temperature changed after (1) subcutaneous injection (A) (15 rats) of 1.0 cc of Higgins India ink; (2) intraperitoneal injection of (B) 0.2 cc (8 rats), (C) 1.0 cc (8 rats), and (D) 2.0 cc (15 rats) of Higgins India ink, and (3) intraperitoneal injection of 2.0 cc of 2.0% suspension of blood charcoal (8 rats).

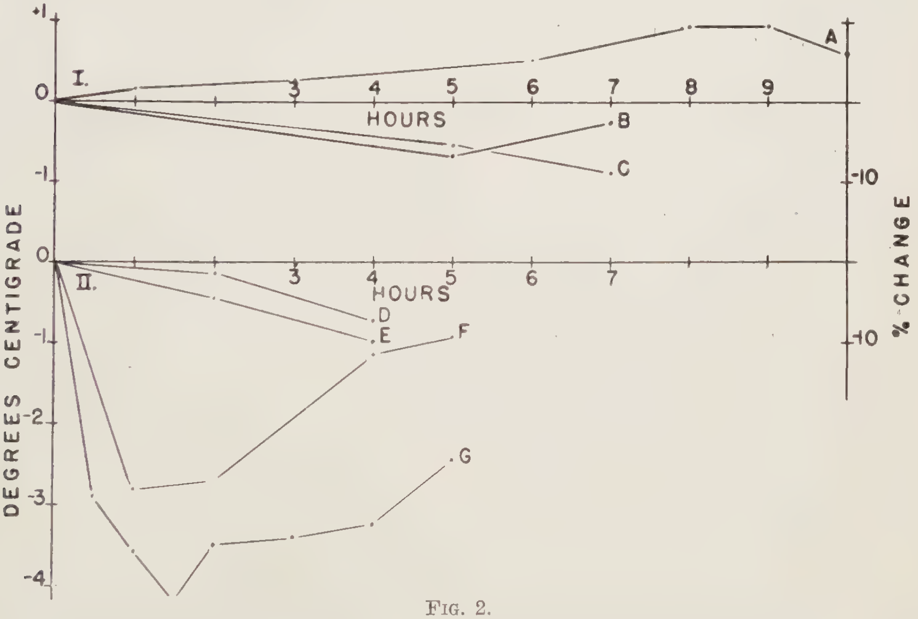

FIG. 2.

I. Average body-temperature change after subcutaneous injection of 4 cc of 1.0% carmine (A). Per cent change in cell volume (hematocrit) shown in C and plasma protein in B (15 rats).

II. Average body-temperature change after intraperitoneal injection of 4 cc (G) (8 rats) and 2 cc (F) (8 rats) of 1.0% carmine. Per cent changes in cell volume are shown in E and plasma protein in D for the rats of curve G.

injection, measurement of body-temperature and the care of the animals were the same as those already described.[1] Young adult white rats were used in all instances.

Experimental Results. Fig. 1 shows average curves of body-temperature change after intraperitoneal administration of 0.2 cc (B), 1.0 cc (C), and 2.0 cc (D), and the subcutaneous administration of 1.0 cc (A) of India ink. In this figure is included a curve (E) that

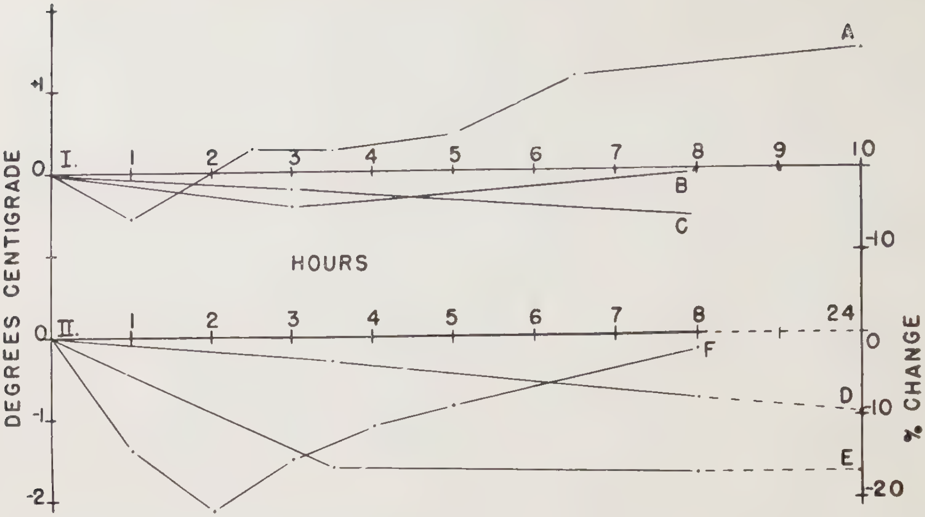

Fig. 3.

I. Average body-temperature change (A) after intramuscular injection of 1 cc of 4% carmine. Average per cent change in cell volume (hematocrit) (B) and plasma protein (C) in the same animals (4 rats).

II. Average body-temperature change (F) after intraperitoneal injection of 1 cc of 4% carmine. Average per cent change in cell volume (D) and plasma protein concentration (E) in the same animals (8 rats).

represents the changes in body-temperature after intraperitoneal administration of 2.0 cc of a 2.0% suspension of blood charcoal.

The expected rise in body-temperature was observed after subcutaneous injection of India ink. After intraperitoneal injection, the body-temperature showed a fall, the degree and duration of which depended upon the amount of ink administered. Dilutions of India ink 1-100 given in 2 cc doses were ineffective; 1-10 dilutions given in the same volumes gave small responses in some animals. These data are not represented in the figure. Blood charcoal given intraperitoneally gave less intense but qualitatively the same response as India ink.

Carmine (Fig. 2) in doses of 4 cc of a 1.0% preparation produced a fever when given subcutaneously (A) but was somewhat less active than 2 cc of India ink. The same dose given intraperitoneally (G) reduced the body-temperature, both qualitatively and quantitatively, much as was done by 2.0 cc of India ink. Intraperitoneal injection (F) of 2.0 cc of 1.0% carmine gave a less prolonged response. The changes in cell volume (subcutaneous injection C; intraperitoneal injection E) and plasma protein concentration (subcutaneous injection B; intraperitoneal injection D) may have been caused by dilution with the water injected. Similar changes followed the injection of saline alone.

The results of intramuscular injection of carmine are shown in Fig. 3 (I). In order to avoid over-distention of the tissue and also to give the same dose of carmine, 1 cc of a 4% preparation was used instead of 4 cc of a 1% preparation. After a short period of hypothermia, a prolonged hyperthermia followed this treatment. The more mild decreases in cell volume and plasma protein concentration after injection of the smaller volumes are consistent with the hypothesis that these phenomena are due to dilution.

In Fig. 3 (II) are shown the results of intraperitoneal injection of 1 cc of the 4% preparation of carmine. This more concentrated preparation given in smaller volume has less effect on body-temperature (F) than 4 cc of the 1% preparation (Fig. 2). At the same time there is a greater fall in the plasma protein concentration (E) and a somewhat greater fall in the cell volume (D). The great fall in plasma protein concentration

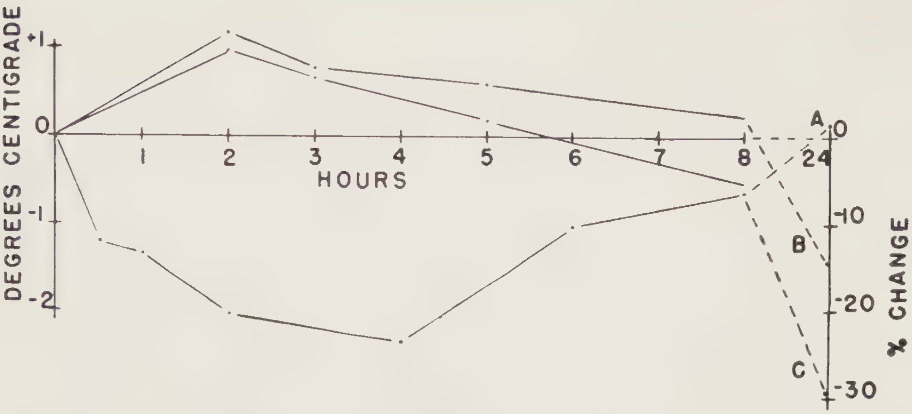

Fig. 4.

Average changes in body-temperature (A) after the intraperitoneal injection of 4 cc of 20% peptone. Per cent change in cell volume (hematocrit) (C) and plasma protein (B) in the same animals (6 rats).

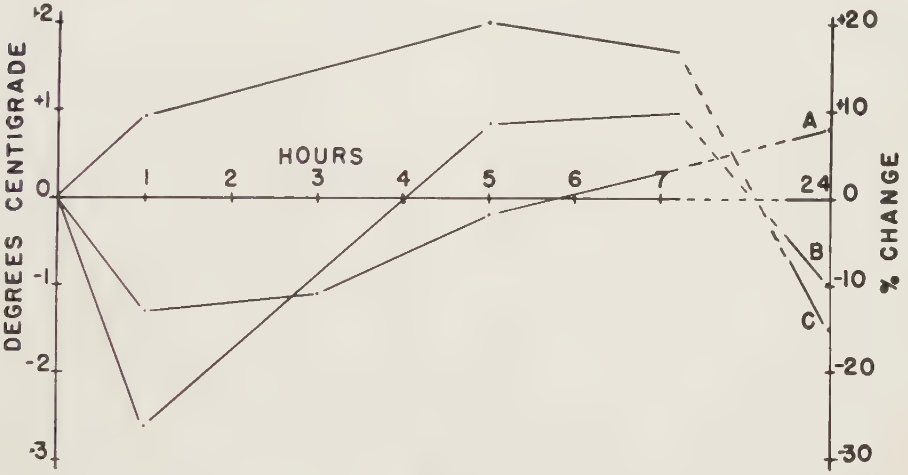

Fig. 5.

Average change in body-temperature (A) after the intraperitoneal injection of 0.5 cc of ox bile. Per cent change in cell volume (hematocrit) (C) and plasma protein (B) in the same animals (6 rats).

(16%) cannot be explained entirely on the basis of dilution. We suggest that the more concentrated carmine preparation contained enough insoluble material that local irritation resulted in mild shock. This, if it is shock, is accompanied, however, not by a more intense but less intense hypothermia.

To save space, no curves are given for the trypan blue experiments. In all cases they were qualitatively like those for carmine; quantitatively, the response was less intense.

Fig. 4 shows curves for body-temperature response and for changes in blood cell volume and plasma protein concentration after intraperitoneal injection of 4 cc of 20% peptone. The decrease in body-temperature (A) is similar to that after 1 cc of India ink or 1 cc of 1% carmine, but is not as great as that after 4 cc of either of these preparations. In spite of the large amount of water administered, there was an early concentration of the blood, as measured by blood cell volume (C) and plasma protein concentration (B), which was followed later by dilution. These changes may be due to an early loss of water into the peritoneal cavity because of hypertonicity of the peptone solution. The fact that the protein and cell volume changes were closely parallel argues against loss of protein into the tissue through the capillary wall. If shock was present in these animals during hypothermia, it was not severe. We cannot explain the dilution of the blood the following day. The animals appeared to be in good condition.

In Fig. 5 are presented the body-temperature changes (A) and changes in the blood cell volume (C) and plasma protein concentration (B) after intraperitoneal injections of 0.5 cc of ox bile. It was found impossible to administer more than this quantity of bile and keep the animals alive as long as was desired. Following bile administration there was a decrease in plasma protein concentration at the same time that the blood was being concentrated as measured by the cell volume. This can be explained as an early local loss of protein through the damaged capillary walls. At the site of protein loss there was presumably a parallel loss of water. In other areas the water was partly restored but the protein could not be supplied as quickly. The net result of loss of water and of protein in one area and partial replacement of water alone in another area is dilution of the circulating plasma protein and a simultaneous concentration of cells. However, later, the plasma protein concentration was increased, and, at the 5th hour was 8% above the initial value. Finally, there was a roughly parallel decrease in both the cell volume and the plasma protein concentration indicating a general dilution of the blood. At autopsy it was observed that engorgement was intense in the splanchnic veins. We believe that the development of shock after intraperitoneal injection of ox bile was unquestionable. However, in spite of the shock, the intensity and duration of the hypothermia was less than was found after injection of the other hypothermia-provoking agents. Gross examination of the peritoneal surfaces after injection of the substances other than bile showed at most only mild reactions.

Discussion. The substances, India ink, blood charcoal, carmine, and trypan blue, whose dual action on body-temperature is described in this paper, have little in common chemically but, as administered, all are particulate. When given intraperitoneally, they are all rapidly taken up by the lymph channels and can be seen in the reticuloendothelial system. However, the fact that a substance is injected in particulate form does not assure that it will affect body-temperature. This is shown particularly well with injections of suspensions of $BaSO_4$ which are entirely inactive. It may be that the particles of $BaSO_4$ are not phagocytized and do not reach the reticuloendothelial system and that this is the reason for its inactivity. On the other hand, no particulate material can be seen in the clear yeast "autolysates" which have a dual effect on body-temperature in the rat as previously described.[1] The very large molecules of yeast nucleic acid, which are presumed to be the active constituent of yeast autolysate, may be handled similarly to particulate material, though we have, as yet, no evidence of this beyond the similarity in thermal reaction after injection.

At present, no explanation can be given for the fall in body-temperature after the intraperitoneal injection of these substances. There is little evidence of shock, and when severe shock is produced by intraperitoneal injection of ox bile, the fall in body-temperature is less than when particulate substances are injected. The cause of hypothermia would seem to lie rather in some interference with the normal functioning of the reticuloendothelial system. Intramuscular injection of carmine produces a rise in body-temperature similar to that following subcutaneous injection. This would seem to rule out differences in blood supply to the area of injection, and consequent differences in the rate of absorption, as the cause of the dual effect of carmine on body-temperature.

Summary. India ink, suspensions of blood charcoal, carmine, and trypan blue were injected subcutaneously and intraperitoneally. Carmine was injected also intramuscularly. After subcutaneous and intramuscular injection a rise in body-temperature was observed. After intraperitoneal injection a fall in body-temperature was observed. As judged by relative engorgement of capillary beds, and changes in blood cell volume (hematocrit) and plasma protein concentration, these animals were not in shock during hypothermia. Intraperitoneal injections of ox bile, and solutions of peptone produced a less intense hypothermia although ox bile caused severe shock.

Received August 8, 1949. P.S.E.B.M., 1949, **72**.

A Highly Active Material that Promotes Conversion of Prothrombokinase Complex.* (17419)

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A procedure has been described[1] for analyzing blood coagulation in three experimental steps: 1. activation of prothrombokinase; 2. activation of prothrombin; 3. coagulation of fibrinogen. Crude thrombokinase, besides activating prothrombin, also accelerated conversion of prothrombokinase. Both activities were lost upon adsorption with barium sulfate.

It is now reported that a material, prepared by elution from barium sulfate, promotes the activation of prothrombin, and also the conversion of crude prothrombokinase. Beginning with a solution of bovine plasma globulins, fraction "A" was adsorbed on Hyflo (Johns-Manville) and eluted with phosphate-potassium chloride solution. The unadsorbed globulins were exposed to adsorption by barium sulfate. From the latter, two successive fractions were eluted, with 0.2-0.3M phosphate, pH 6.6, and with 0.6M phosphate, pH 7.9. The pH 6.6 eluate had more prothrombin. From the pH 7.9 eluate, proteins soluble in 0.35, but precipitable by 0.45 saturated ammonium sulfate were selected as the "converter fraction." This involved 3 extractions and 20 precipitations. Proteins soluble in 0.45, but precipitable by 0.60 saturated ammonium sulfate, constituted the "thrombin fraction" (3 extractions, 7 precipitations). Fifty-five liters of plasma yielded 10 ml of each fraction. Both were dialyzed simultaneously in the same beaker.

Activations were studied with calcium present, giving results summarized in Table I. At high dilution, the converter hastened both the activation of prothrombin and the conversion of crude prothrombokinase. In comparing different preparations, converter activities estimated by the 2-stage and 3-stage procedures were roughly parallel. This is compatible with the view that the tests measured two different effects of the same substance.

In contrast, converter activity did not parallel thrombin activity. The ratio of converter to thrombin activity was 100 times as great in the converter fraction as in the thrombin fraction. Conceivably, varieties of thrombin can exist with different converter activities, or some factor modifies the behavior of thrombin in one of the fractions. If, as must be contemplated, the converter is distinct from thrombin, the results suggest how difficult would be its elimination from thrombin by salting-out. Moreover, the converter is manifest in sufficiently minute amount to cause confusion, even as a small contamination in a highly purified thrombin.

The relation of "A" and converter to thrombokinase activity resembles that reported for platelets plus "globulin".[2] Their effects are complementary, but not simply additive. Clear evidence has not been obtained that their mutual reinforcement is due chiefly to progressive activation of one by the other. An additional possibility must be considered, that one is thrombokinase and the other contains a co-factor or anti-inhibitor.

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* Aided by a grant from the James Hudson Brown Memorial Fund of the Yale University School of Medicine.

[1] Milstone, J. H., *J. Gen. Physiol.*, 1948, **31**, 301.

[2] Milstone, J. H., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 225.

TABLE I.
Effective Amounts of Converter Fraction.

2-stage tests		3-stage tests
Activate half of prothrombin in 40 min.	Accelerate prothrombin activation in presence of aged fraction "A"	Accelerate conversion of crude prothrombokinase
2/11 dilution	1/1,000 dilution 1 μg protein-N/ml	1/45,000 dilution 0.02 μg protein-N/ml

Figures refer to final dilution or concentration in the respective activation mixtures. A concentration of 0.02 μg protein-N per ml prothrombokinase mixture becomes 0.002 μg per ml when the mixture is diluted tenfold in prothrombin. The tests were performed as previously described.[1,2]

Previous comparison[1] of prothrombokinase to Factor V was based on Owren's tentative *concept* of V as the precursor of VI.[3] If his V *preparation* did not so function, the comparison does not apply to it. Crude prothrombokinase "may contain an activator complex with more than one significant component."[1] More than one transformation may occur in the complex.

Although the converter fraction is highly active, it may be far from pure, chemically. It *may* represent a thrombokinase which activates not only prothrombin, but also prothrombokinase. Several years ago,[4] it was reported that concentrated prothrombin changed to thrombin in the absence of ionic calcium, without addition of activators; and the possibility was left open that a contaminant was responsible. The possibility must be entertained that the converter contaminates highly purified clotting preparations, and might be responsible for certain effects occasionally attributed to prothrombin or thrombin.

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[3] Owren, P. A., *Acta Med. Scand.*, 1947, suppl. 194.

[4] Milstone, J. H., *J. Gen. Physiol.*, 1942, **25**, 679.

Received August 9, 1949. P.S.E.B.M., 1949, **72**.

A Modification of the Hemagglutination Test for Rheumatoid Arthritis.* (17420)

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A diagnostic test for rheumatoid arthritis based upon the agglutination of sheep erythrocytes has been recently reported by Rose, Ragan, Pearce and Lipman.[1] Serum from patients with rheumatoid arthritis agglutinates sheep erythrocytes sensitized with sheep cell hemolysin to a titer at least 16 fold higher than that obtained with normal sheep cells. These are expressed as a differential titer

$$\left(\frac{\text{titer with sensitized cells}}{\text{titer with normal cells}}.\right)$$

The test has been confirmed but may be of limited value for diagnostic purposes.[2,3] In the performance of the test as described by Rose, *et al.*,[1] we have found that the factor in the serum responsible for the agglutination of normal sheep erythrocytes was present in the sera of both normal and rheumatoid arthritic individuals. This normal agglutinin is a variable factor and is independent of the capacity of the sera of patients with rheumatoid arthritis to agglutinate sensitized cells. It can be selectively absorbed without significantly influencing the titer against sensitized sheep cells. The titer thus obtained reflects the concentration of the factor associated with rheumatoid arthritis. At the same time the need for expressing the results of the test as a differential titer is eliminated. Based upon an absorption technic, a modification of the original test is herein described. A comparison of the results of the test as modified by us, the test as originally described by Rose, *et al.*,[1] and the streptococcus agglutination test is presented.[4–6]

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* Published with the permission of the Chief Medical Director, Department of Medicine and Surgery, Veterans Administration, who assumes no responsibility for the opinions expressed or the conclusions drawn by the authors.

[1] Rose, H. M., Ragan, C., Pearce, E., and Lipman, M. O., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 1.

[2] Sulkin, S. E., Pike, R. M., and Coggeshall, H. C., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 475.

[3] Jawitz, E., and Hook, E. V., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 650.

Method and procedure. 1. *Preparation of Normal Sheep Erythrocytes.* One volume of sheep blood is mixed with 1.2 volumes of Alsever solution. Sufficient formaldehyde is added at collection to make a final concentration of 0.2%. This mixture stored at 4-6°C will usually last 4 to 6 weeks. The blood-Alsever mixture should be kept in the refrigerator for 2-3 days before use. The cells are washed 3 times in a graduated centrifuge tube with saline, centrifuging at 2000 rpm for 15 minutes between each washing. The supernatant of the last washing must be water clear. The packed cells are used for absorption. A 2% suspension in saline is also prepared from packed cells. Part of this is further diluted with saline to make a 1% suspension for use in the test.

Preparation of Sensitized Sheep Erythrocytes. Rabbit anti-sheep-erythrocyte hemolysin is prepared in concentrations of 1:4000 (2 units per cc) determined by the complement-fixation technic.[1] To the 2% suspension of erythrocytes as described above an equal volume of a 1:4000 dilution of amboceptor is added. The solution should be allowed to stand for at least 10 minutes before use.

Treatment of patient's serum. Clear serum is obtained from clotted blood and inactivated at 56°C for one-half hour. Serum may be stored in a deep freeze at –20°C for at least 5 months without significant loss of potency.[7] For absorption, 4 volumes of serum are mixed with 1 volume of packed sheep erythrocytes and allowed to remain at room temperature for 40 minutes with gentle shaking at intervals. The mixture is centrifuged and the clear serum is decanted into an additional volume of packed cells for another 40-minute incubation period at room temperature. The mixture is again centrifuged and the clear serum is used for the test. Two absorptions have been adequate to eliminate the normal agglutinating factor for normal sheep cells. Additional absorptive treatment is necessary in cases where the heterophile antibody associated with infectious mononucleosis is present. For this purpose, a 20% boiled ox erythrocyte suspension[8] is centrifuged and the supernatant fluid discarded. Four volumes of the serum is mixed with one volume of the packed sediment, incubated at 37°C for 1 hour, centrifuged, and the serum removed.

Procedure. Two series of two-fold dilutions of absorbed serum are made in 0.5 ml volumes of normal saline. In the first series, consisting of three tubes, the serum dilutions range from 1:3.5 to 1:14. To this series is added an equal volume (0.5 ml) of 1% normal sheep cells; the final dilution range is thus 1:7 to 1:28. The second series, consisting of 10 tubes, is similarly diluted (1:3.5 to 1:1792). To this series, 1% sensitized sheep cells are added in 0.5 ml volume, thus making the final dilutions 1:7, etc. The tubes are shaken to make a homogeneous suspension of the cells, and incubated in a water bath at 37°C for one hour. The tubes are then refrigerated overnight. Readings are made after gentle

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[4] Cecil, R. L., Nicholls, E. E., and Stainsby, W. J., *Arch. Int. Med.*, 1929, **43**, 571.

[5] Cecil, R. L., Nicholls, E. E., and Stainsby, W. J., *Am. J. Med. Sc.*, 1931, **181**, 12.

[6] Lipman, M. O., Laboratory of the Edward Daniels Faulkner Arthritis Clinic, Presbyterian Hospital, New York, personal communication.

[7] Unpublished data.

[8] Kolmer, J. A., and Boerner, F., Approved Laboratory Technic, D. Appleton-Century Co., N. Y., 4th ed., 1945, p. 644.

TABLE I.
Comparison of Agglutination Titers Obtained with Unabsorbed and Absorbed Sera from Representative Cases.

Diagnosis	Rose test			Modified test	
	Unabsorbed serum +			Absorbed serum +	
	Normal RBC	Sensitized RBC	Differential titer	Normal RBC	Sensitized RBC
Active peripheral rheumatoid arthritis (5 cases)	56	896	16	0	896
	56	1792	32	0	1792
	0	448	448	0	224
	14	112	8	0	224
	56	448	8	0	448
Rheumatoid spondylitis with active peripheral joint involvement (4 cases)	112	224	2	0	112
	0	896	896	0	1792
	14	1792	128	0	896
	0	896	896	0	1792
Rheumatoid spondylitis without peripheral joint involvement (3 cases)	7	7	1	0	0
	14	28	2	0	0
	28	56	2	0	0
Psoriatic arthritis (2 cases)	14	14	1	0	0
	7	14	2	0	0
Cirrhosis of liver	56	112	2	0	0
Hyperglobulinemia (2 cases)	14	14	1	0	0
	28	56	2	0	0
Brucellosis	7	14	2	0	0
Duodenal ulcer	0	14	14	0	0
Gout	14	28	2	0	0
Osteoarthritis	7	28	4	0	0
Rheumatic polyarthritis	28	28	1	0	0

RBC refers to sheep red blood cells.

shaking. For simplicity, 3 values are used: complete agglutination, partial agglutination, and no agglutination perceptible to the naked eye. The test is valid only if no agglutination is observed in the tubes containing the normal cells, thereby indicating complete absorption of the normal sheep cell agglutinin. Both the Rose test and the modified test were run concurrently; the same reagents were always used for both tests. The Rose test was performed as described[1] except that the serial dilutions were the same as those used in our modified test. The streptococcus agglutination test was also performed as described by Lipman.[6]

Results. Agglutinins for normal sheep erythrocytes (Forssman antibody) may be found in the sera of normal individuals as well as in those with rheumatoid arthritis. In our studies, it was found that sera obtained at intervals from the same patient showed variations in the normal agglutinin titer.[7] It is not yet clear whether these variations reflect a fluctuating agglutinin content or are associated with the varying agglutinability of different batches of sheep erythrocytes. Table I demonstrates that this normal agglutinin can be completely absorbed with normal sheep cells. As a result of this procedure, only the sera from patients with active peripheral rheumatoid arthritis retained the capacity to agglutinate sensitized cells. The sera from patients with diseases other than rheumatoid

TABLE II.
Differential Absorption of the Heterophile Factor from Serum of Patients with Infectious Mononucleosis and Patients with Rheumatoid Arthritis.

Diagnosis	Titer of unabsorbed serum +			Titer of serum absorbed with normal sheep cells +		Titer of serum absorbed with sheep and ox cells +	
	Normal RBC	Sensitized RBC	Differential titer	Normal RBC	Sensitized RBC	Normal RBC	Sensitized RBC
1. Infectious mononucleosis	896	1792	2	112	448	0	0
2. Rheumatoid arthritis	14	448	32	0	448	0	448
3. Mixture of equal vol. of No. 1 and 2	448	896	2	224	896	0	224
4. Infectious mononucleosis	896	896	1	112	448	0	0
5. Rheumatoid arthritis	28	3584	128	0	1792	0	1792
6. Mixture of equal vol. of No. 4 and 5	448	896	2	112	1792	0	1792

RBC refers to sheep red blood cells.

arthritis (control group) gave no significant agglutination. Removal of the normal (non-specific) factor by absorption did not materially affect the titer of the factor associated with rheumatoid arthritis. The sera from cases of infectious mononucleosis could not be completely absorbed by the routine method described. The heterophile antibody continued to give a positive titer with normal sheep cells after absorption of the sera with sheep erythrocytes. However, additional absorption of the sera with boiled ox cells resulted in complete absorption of the heterophile factor. In cases of active peripheral rheumatoid arthritis, such multiple absorptions did not affect the final titer. Mixtures of sera from cases of rheumatoid arthritis and cases of infectious mononucleosis, when treated by this method and allowing for dilutions, retained only the titer of the rheumatoid arthritis factor (Table II).

Table III shows the distribution of agglutination titers using the modified test in 166 cases. Only cases with active peripheral rheumatoid arthritis (with the exception of 2 cases of infectious hepatitis) gave titers of 1:28 or higher with complete agglutination in the first 2 dilutions. For this reason, this value is considered to be the lower limit of a positive reaction. Using this criterion, 35 of 39 cases (90%) of active peripheral rheumatoid arthritis (8 of which also had spondylitis) gave positive values ranging from 1:28 to 1:3584. All 39 were unequivocal cases of active peripheral rheumatoid arthritis with x-ray evidence of small joint involvement. Negative reactions were obtained with the sera of all cases of inactive[†] peripheral rheumatoid arthritis, rheumatoid spondylitis without peripheral joint involvement or with inactive peripheral joint involvement, and in all cases of psoriatic arthritis. A group of 13 cases of arthritis in which the clinical course was not inconsistent with the picture of early peripheral rheumatoid arthritis but in which x-rays showed no evidence of joint involvement were all negative. All other forms of arthritis, non-rheumatoid in type, and the non-arthritic controls were negative. Ten cases of infectious hepatitis were tested. No determination could be made in one of these cases by our method because complete absorption could not be obtained. After repeated absorptions (10 times), the titer against unsensitized cells remained 1:28. In two other cases the titers were positive (Table III).

[†] A case was considered inactive if the patient was clinically in symptomatic remission with absence of swelling, pain and tenderness of involved joints and with the sedimentation rate normal.

TABLE III.
Distribution of Cases According to Agglutination Titer, Using the Modified Test.

Diagnosis	No. of cases	Agglutination titer										
		0	7	14	28	56	112	224	448	896	1792	3584
Active peripheral rheumatoid arthritis												
Without spondylitis	31			1	2(a)	2	3	4	8	5	5	1
With spondylitis	8	2			2		1			1	2	
Peripheral rheumatoid arthritis of questionable activity (1)	7	3		2	1(b)	1						
Inactive peripheral rheumatoid arthritis	8	5	1	1	1(b)							
Rheumatoid spondylitis without peripheral joint involvement	15	12	1	2								
Rheumatoid spondylitis with inactive peripheral joint involvement	7	5	2									
Psoriatic arthritis	9	6	1	2								
Arthritis, type undetermined, possibly rheumatoid (2)	13	10	2	1								
Other arthropathies (3)	30	27	2	1								
Non-arthritic controls except infectious hepatitis (4)	28	23	3	1	1(b)							
Infectious hepatitis	10(c)	3	2	2	1	1						

(1) Definitive evaluation of activity could not be made on the basis of clinical impression and laboratory data.

(2) Clinical course was not inconsistent with early rheumatoid arthritis, but x-ray studies showed no changes in affected joints.

(3) These include 10 cases of rheumatic polyarthritis, 7 cases of gout, 2 cases of osteoarthritis, and 11 cases of arthritis following a variety of infection.

(4) Among these are included 3 cases of hyperglobulinemia and 4 cases of infectious mononucleosis.

(a) One of these is considered negative because the results showed incomplete agglutination in first two tubes.

(b) Negative because of incomplete agglutination in first two tubes.

(c) Determination of titer in 1 case of infectious hepatitis could not be made because of incomplete absorption of the non-specific factor by the standard technic.

Table IV presents a summary of the results obtained with the sera of 166 patients by the modified test, the original Rose technic, and the streptococcus agglutination test. In the group of 39 patients with active peripheral rheumatoid arthritis of varying severity and duration, eight of whom also had spondylitis, the modified test gave a significantly higher percentage of positive reactions than either of the other two tests. Both the modified and the Rose tests were consistently negative in inactive peripheral rheumatoid arthritis, active or inactive spondylitis without peripheral joint involvement, and in psoriatic arthritis. In these groups, the streptococcus agglutination test, however, was found on several occasions to be positive. The group of 13 cases of arthritis in which no definitive diagnosis could be made were negative to all 3 tests employed. In all other arthropathies, including osteoarthritis, rheumatic polyarthritis, gout, *etc.*, the modified test and the Rose test were consistently negative. This was also true of the non-arthritic controls with the exception of infectious hepatitis in which two were positive by our test but negative by the Rose test.

Table V presents the findings in the 11 cases of active peripheral rheumatoid arthritis in which the Rose test was negative whereas the modified test was positive. It is to be noted that in each case the titer against the sensitized sheep red cells was significantly elevated but the differential titer in the Rose test fell below 16 because of the presence of a relatively high non-specific agglutinin titer. This non-specific factor was removed in the

TABLE IV.
Summary of Results Comparing Tests Employed.

Diagnosis	No. of cases	Modified test (1)		Rose test (2)		Streptococcus agglutination	
		Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
Active peripheral rheumatoid arthritis							
Without spondylitis	31	29	2	21	10	18	13
With spondylitis	8	6	2	3	5	5	3
Total	39	35	4	24	15	23	16
%	(100)	(90)	(10)	(61)	(39)	(58)	(42)
Peripheral rheumatoid arthritis-questionable activity	7	1	6	1	6	2	5
Inactive peripheral rheumatoid arthritis	8	0	8	0	8	2	6
Rheumatoid spondylitis without peripheral joint involvement	15	0	15	0	15	3	12
Rheumatoid spondylitis with inactive peripheral rheumatoid arthritis	7	0	7	0	7	0	7
Psoriatic arthritis	9	0	9	0	9	1	8
Arthritis, type undetermined, possibly early rheumatoid arthritis	13	0	13	0	13	0	13
Other arthropathies (3)	30	0	30	0	30	2	28
Non-arthritic controls except infectious hepatitis (4)	28	0	28	0	28	Not done	
Infectious hepatitis	9	2	7	0	9	1	8

(1) A titer of 28 or higher with complete agglutination in first 2 tubes is considered positive.
(2) A differential titer of 16 or greater is considered positive.
(3) See Note No. 3—Table III.
(4) See Note No. 4—Table III.

modified test, and the resulting titer was positive in each case. No sera were encountered in which the Rose test was positive and our modified test negative. It is of interest that in the 4 cases of active peripheral rheumatoid arthritis which were negative by our test, both the Rose and the streptococcus agglutination tests were also negative.

Discussion. The agglutination of unsensitized sheep cells by human sera is due to a Forssman antibody (natural amboceptor).[9] This antibody may be present in varying concentrations or may occasionally be absent. It is not related to the hemagglutinin associated with active rheumatoid arthritis nor is it necessary for the reaction with sensitized cells. The factor in the sera of patients with rheumatoid arthritis responsible for the agglutination of sensitized sheep cells behaves like an antibody. This is suggested by its reaction in selective absorption studies and its identity as part of the beta-gamma globulin complex.[1] Our results indicate that this substance is different serologically from the natural amboceptor of human serum as well as from the heterophile antibody of infectious mononucleosis.[7] Evidence of its specificity is found

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[9] Kabat, E. A., and Mayer, M. M., Experimental Immuno-chemistry, Charles C. Thomas, Springfield, Ill., 1948, p. 135.

TABLE V.
Comparative Results in Those Cases of Active Rheumatoid Arthritis in Which the Rose Test Was Negative and the Modified Test Positive.

	Rose test			
Diagnosis	Normal RBC	Sensitized RBC	Differential titer	Modified test titer
Active peripheral rheumatoid arthritis (8 cases)	28	112	4	56
	7	56	8	28
	14	56	4	28
	28	224	8	224
	112	224	2	56
	14	112	8	224
	56	448	8	448
	14	112	8	448
Active peripheral rheumatoid arthritis with spondylitis (3 cases)	7	28	4	28
	112	224	2	112
	28	56	2	28

RBC refers to sheep red blood cells.

in the fact that it is present in a high percentage of proven cases of rheumatoid arthritis and cannot be demonstrated in any of the other arthritides or in a wide variety of unrelated medical controls. The occurrence of positive reactions in 2 cases of infectious hepatitis, both in low titer, may be due to the presence of altered globulins or unabsorbed antibodies associated with this disease. The concentration of rabbit anti-sheep amboceptor used in our test is based on its titer as determined by complement-fixation.[1] Our experience has shown that varying the concentration within critical limits will produce false reactions.[7] We believe the present method of determining the sensitizing unit of amboceptor is unsatisfactory. Standardization of the sensitizing unit should be based on the capacity of a particular pool of amboceptor to agglutinate a particular suspension of sheep cells rather than on its lytic capacity. Further studies on standardization along these lines are being pursued.

Natural amboceptor in human sera can contribute to the further sensitization of cells thereby increasing their agglutinability. When the rheumatoid arthritis hemagglutinin is present in high titer, the amount of naturally occurring amboceptor is not of practical significance because of dilution. For this reason there is no essential difference in the titer obtained by absorbed and unabsorbed sera against sensitized cells (Table I). However, when the rheumatoid arthritis hemagglutinin is of relatively low titer, the normal amboceptor in the unabsorbed serum exerts an additive effect with the rabbit anti-sheep amboceptor. In this event, there is relatively little dilution and the titer may then be higher than that obtained by absorbed serum (Table V). Based upon our data, a titer of 1:28 with complete agglutination in the first two tubes can be considered positive. Thus far, we have found that the concentration of the rheumatoid arthritis factor does not necessarily correlate with clinical severity of the disease. On the other hand, in no case of inactive disease was a positive titer obtained. A serious limitation of the test as a diagnostic tool is that it appears to become positive only when x-rays begin to show the joint changes characteristic of rheumatoid arthritis. In the group classified as arthritis of unknown type in which the course was not inconsistent with rheumatoid arthritis but where no x-ray changes characteristic of the disease were present, the test was negative. It will be noted that the streptococcus agglutination test and the Rose test were also negative in all of these cases.

If the hemagglutinin associated with rheumatoid arthritis is indeed an antibody, investigation is required to determine whether this antibody is the result of immunization by an etiological agent or whether it is analogous to the non-specific antibody of the complement-fixation reaction which is almost always associated with syphilis. If it is a specific antibody, its absence in all cases of active rheumatoid spondylitis without peripheral joint involvement and in psoriatic arthritis is suggestive of different etiologies. The modified test, by eliminating the natural amboceptor and by more closely reflecting the titer of the rheumatoid arthritis factor, is more sensitive than the original hemagglutination test. A comparison of the 3 tests employed indicates that a significantly higher incidence of positive reactions can be obtained by this procedure.

Summary. 1. The hemaggutination test for rheumatoid arthritis is modified by the selective absorption of non-specific factors. The details of this modification are described.

2. Of 39 cases with active peripheral rheumatoid arthritis with x-ray evidence of joint changes, 35 were positive by this test.

3. All cases of inactive peripheral rheumatoid arthritis, rheumatoid spondylitis without peripheral joint involvement and psoriatic arthritis tested were negative.

4. In presumptive cases of early rheumatoid arthritis without x-ray evidence of joint changes, the test was negative.

5. In 67 control cases, including a variety of non-rheumatoid arthropathies, the test was negative except in two cases of infectious hepatitis.

6. The modified test was positive in 90% of the proven cases of active peripheral rheumatoid arthritis tested; the Rose test in 61%; the streptococcus agglutination test in 58%. In all cases in which the modified test was negative, the Rose test and the streptococcus agglutination test were also negative.

The authors express their thanks to Dr. R. Schuman, Miss E. C. Arrowsmith, and Mr. O. Vasquez of the Clinical Laboratory Service for technical assistance.

Received August 12, 1949. P.S.E.B.M., 1949, **72**.

Methyl Alcohol Purification of the Rabbit Papilloma Virus.*† (17421)

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Rabbit papillomatosis is an endemic virus disease of cottontail rabbits readily transmissible to domestic rabbits. Serum from infected cottontail and domestic rabbits contains antibody which is capable of neutralizing the virus *in vitro*.[1] The virus can be obtained easily from cottontail rabbit papillomas, but ordinarily it is not recoverable from domestic rabbit growths.

The demonstration that extravasated fluid from large confluent cottontail papillomatous growths was effective in neutralizing active virus led Kidd[2] to suggest that the neutralizing principle represented antibody derived through escape from altered capillaries. Kidd[2] found no evidence of such "masking" in naturally occurring papillomas of cottontail rabbits.

When Kidd[2] obtained no virus from the growths of 8 of 11 domestic rabbits and little from the other 3, and found that the sera of the 11 rabbits had little or no neutralizing capacity, he concluded that it was improbable that antibody was primarily responsible for the masking of the virus in the growths produced in domestic rabbits.

The method developed by Cox, van der Scheer, Aiston and Bohnel[3] for the purification and concentration of influenza and other

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* This investigation was supported by a grant from the American Cancer Society, on recommendation of the Committee on Growth, National Research Council.

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[1] Shope, R. E., and Hurst, E. W., *J. Exp. Med.*, 1933, **58**, 607.

[2] Kidd, J. G., *J. Exp. Med.*, 1939, **70**, 583.

viruses by employing organic solvents at low temperatures to precipitate virus proteins led us to explore this technic in our efforts to characterize the agent responsible for the masking of the papilloma virus. Accordingly, experiments were designed to isolate the virus of infectious rabbit papillomatosis by precipitation (a) from crude tissue suspensions derived from naturally occurring cottontail papillomas, (b) from a suspension of virus artificially "masked" as the result of specific neutralization *in vitro,* and (c) from tissue suspensions derived from virus-induced domestic rabbit papillomas.

It is the purpose of this paper to report the results of our findings.

Methyl Alcohol Purification of Cottontail Papilloma Tissue. Four grams of cottontail papilloma tissue were ground with sand in a mortar to yield a 20% suspension in saline. After centrifugation in an angle centrifuge at 5,000 r.p.m. for thirty minutes at 4°C, the supernatant fluid was withdrawn. Ten ml of ether were added to 20 ml of the supernatant material. The mixture was shaken and centrifuged at 5,000 r.p.m. for 30 minutes at 4°C. After withdrawal of the lipid layer the remaining ether was removed *in vacuo.* The suspension was frozen rapidly at -20°C, thawed at room temperature, and centrifuged at 2,500 r.p.m. for 30 minutes at 4°C. The supernatant was drawn off, diluted to 200 ml with 0.1 M phosphate buffer, pH 7, cooled in a dry ice bath, and absolute methyl alcohol at –40°C was added drop by drop by means of a capillary pipette with constant stirring until the alcohol concentration reached 25%. The suspension was then kept in an ice bath for one hour at –2°C to allow time for the formation of the precipitate. The material was centrifuged at 5,000 r.p.m. for 30 minutes at 4°C. The alcohol was decanted and the precipitate washed with .02 M phosphate buffer, pH 5.1, to remove more nonviral protein. The addition of 4 ml of phosphate buffer, pH 7.0, to the resultant precipitate made available papilloma virus in a suspension of which 1 ml was equivalent to 1 gm of tissue. After elution for 15 minutes, the suspension was frozen, thawed, and centrifuged at 2,500 r.p.m. for 30 minutes. The final water-clear supernatant fluid was kept in storage at –20°C until used.

Tests for the Presence of Virus in Purified Material. The purified viral suspension was diluted so as to have 8 successive two-fold dilutions ranging from 1-10 to 1-5120 to test for infectivity by transfer to domestic rabbits. Each dilution was inoculated in triplicate by the application of 0.2 ml by crosshatch scarification to previously shaved areas of skin on 3 rabbits. The results as observed 4 weeks later are recorded in Table I.

For control purposes to learn the infectious titer of the papillomatous tissue which was employed for methanol precipitative purification, infectivity tests in triplicate of the non-purified virus suspension in dilutions of from 1-10 to 1-6400 were carried out. Papillomatous growths were present 4 weeks later at all sites of inoculation.

It appears from these results that virus was lost in the process of purification, since the limiting dilution for infectivity of the purified virus was found to be 1-2560, whereas an end point was reached for the control material in a dilution of 1-6400.

These findings were established when the same technic for purification, concentration and biologic testing of 5 additional samples of cottontail papilloma tissue that had been collected between 1938 and 1944 yielded similar results. In each instance the purified virus suspension produced growths in dilutions through 1-2560 and the control virus suspensions yielded tumors in slightly higher dilutions, in most cases 1-6400.

Methyl Alcohol Purification of Neutralized Papilloma Virus. Cottontail papilloma tissue that had been stored in 50% glycerin at 4°C was ground to a fine paste in a mortar, and saline was added to make a 20% suspension. This was centrifuged at 2,000 r.p.m. for 20 minutes, and the supernatant material removed. Blood was drawn by cardiac puncture from a domestic rabbit which had borne several large papillomatous growths for about 3 months, and the serum was separated by centrifugation. Ten ml of serum was added to

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[3] Cox, H. R., van der Scheer, J., Aiston, S., and Bohnel, E., *J. Immunol.*, 1947, **56**, 149.

TABLE I.
Growths Produced with Purified Virus.

Rabbit	Reciprocal of dilution 10	40	160	320	640	1280	2560	5120
1	+	+	+	+	+	+	+	—
2	+	+	+	+	+	+	+	—
3	+	+	+	+	+	+	+	—

(+) Visible papillomatous growth.
(—) No visible growth.

an equal volume of the 20% viral suspension. The mixture was incubated at 37°C for one hour followed by refrigeration at 4°C overnight. For control purposes a 20% suspension of unneutralized virus was treated in the same manner. Following the incubation and refrigeration, 20 ml of the neutralized virus were subjected to the methanol purification procedure described previously. Four ml of phosphate buffer, pH 7.0, was added to the final precipitate. This suspension was allowed to elute for 15 minutes, frozen, thawed, and centrifuged at 2,500 r.p.m. for 30 minutes. The final supernatant represented the test inoculum. Three normal rabbits were prepared for inoculation by shaving and lightly scarifying 3 dermal areas. Each rabbit was then inoculated by cross-hatch scarification with the methyl alcohol purified neutralized virus in 0.2 ml amounts, and with control suspensions of neutralized virus and of crude virus. The control suspension had been carried through the same incubation and refrigeration procedures as the methyl alcohol purified material. After an incubation period of 5 weeks the rabbits were examined for signs of papillomatous growth. No growth appeared on any of the areas inoculated with the neutralized virus or with the methyl alcohol purified neutralized virus. Control inoculations of virus alone resulted in confluent papillomatosis.

The results indicated that methanol purification was ineffective for the separation of infective virus from the virus-antibody combination, since no visible papillomatous growths appeared in 6 rabbits after inoculation with the purified material. Three additional experiments failed to show that the virus could be extracted from the combination.

Methyl Alcohol Purification of Domestic Rabbit Papilloma Tissue. Papilloma tissue was removed surgically from pure-bred Dutch domestic rabbits which had borne the growths for 6 weeks or more. The tissue was triturated with sand in saline to make a 20% suspension. This material was centrifuged at 5,000 r.p.m. for 30 minutes at 4°C. The supernatant material was drawn off and subjected to the same methyl alcohol purification procedure as was used on the cottontail papilloma tissue. A precipitate was obtained similar to that obtained with the purified cottontail papilloma tissue. Four areas on the ears and flanks of 4 normal pure-bred Dutch rabbits were inoculated by scarification with the purified material. The rabbits were examined for signs of growth at weekly intervals following the inoculation.

When no growths resulted on any of the rabbits inoculated over a 12-week period, it was concluded that the domestic papillomatous growths following treatment by the methanol technic for purification yielded insufficient active virus to cause visible papillomatous growths. Further attempts were made to extract virus from 4 samples of domestic papilloma tissue from rabbits which had borne the growths from 3 to 15 weeks. In no case was any demonstrable virus obtained.

Summary. The methanol precipitative technic of Cox, *et al.*, when applied to the purification and concentration of papilloma virus from naturally occurring cottontail rabbit papillomas, domestic rabbit papillomas and neutral virus-antibody mixtures readily yielded active papilloma virus from the cottontail papillomas but it did not yield virus from either domestic rabbit papillomas or from papilloma virus and its antibody in neutral mixture.

Received August 12, 1949. P.S.E.B.M., 1949, **72**.

Ineffectiveness of Racemic Glyceric Aldehyde in the Prevention of Experimental Tooth Decay. (17422)

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The striking ability of racemic glyceric aldehyde to inhibit acid production by the oral flora during the incubation of saliva was demonstrated in the experiments of Fosdick and Calandra.[1] This observation suggested the need to investigate the ability of dl-glyceric aldehyde to influence the initiation and development of carious lesions in experimental animals of known caries-susceptibility. In an earlier series of experiments conducted in this laboratory, additions of 0.5%, 1.0% and 2.0% crystalline racemic glyceric aldehyde to the caries-producing purified ration throughout the experimental period had no effect on the dental caries incidence of white rats and cotton rats.[2] In the above tests, the glyceraldehyde was present in the ration in the dimeric form and, presumably, passed through the oral cavity largely, if not entirely, as the dimer. Since the glyceraldehyde used in the experiments of Fosdick and Calandra was in solution, where some dissociation of the dimer to the monomer might have occurred, experiments with caries-susceptible animals were undertaken to determine if racemic glyceraldehyde given throughout the experimental period as the monomer influenced the dental caries experience.

Experimental. Six litters of white rats were used as the subjects of the first experiment. These animals were born to females which had been maintained throughout their reproductive lives on purified ration 100.[3] Previous litters from the same females had had a high caries-susceptibility. The 35 weanling rats from these litters were distributed into 3 groups. The 12 rats in the first group were fed ration 100 plus 10% Cellu flour and allowed to drink tap water. The second group of 9 rats was offered the above ration supplemented with 0.5% crystalline dl-glyceric aldehyde and drank tap water. The third group of 14 rats was fed ration 100 plus 10% Cellu flour and was only allowed to drink tap water to which 0.2% dl-glyceric aldehyde had been added.

In the second experiment a litter of 6 weanling cotton rats was divided into 2 equal groups. The cotton rats in the first group served as controls and were offered ration 100 plus 10% Cellu flour and tap water; those in the second group were fed the same ration but 0.2% dl-glyceric aldehyde was incorporated into the drinking water.

In both experiments, food and drinking water were provided *ad libitum* and the rodents were housed in individual screen bottom cages. The dl-glyceric aldehyde solution was prepared sufficiently in advance of its use to permit dissociation of the dimer to the monomer. Tap water and the 0.2% solution of glyceraldehyde were offered to the animals in identical watering bottles. The 0.2% level of dl-glyceric aldehyde in the drinking water was selected on the basis of a comparison between the average food and water consumptions of these rodents. This value permitted the consumption of approximately the same amount of glyceraldehyde as the 0.5% supplement to the purified ration.

The first experiment was terminated at the end of 20 weeks and the second experiment at the end of 14 weeks. After fixation of the skulls in 95% ethanol, the molar teeth were examined to determine the number and size of the carious lesions.

Results. The average rates of growth of the white rats and cotton rats fed dl-glyceric aldehyde either in the ration or in the drinking water were the same as the average rates of growth of comparable control animals.

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[1] Fosdick, L. S., and Calandra, J. C., *J. Dental Research*, 1947, **26**, 309.

[2] Shaw, J. H., *J. Dental Research*, 1948, **27**, 727; *ibid.*, to be published.

[3] Shaw, J. H., *J. Dental Research*, 1947, **26**, 47.

TABLE I.
Caries Incidence of Rodents Fed Racemic Glyceric Aldehyde in the Ration or in the Drinking Water.

% glyceric aldehyde in supplement	No. of animals	No. of carious molars			No. of carious lesions			Extent of carious lesions		
		Avg	S.E.M.*	C.R.†	Avg	S.E.M.*	C.R.†	Avg	S.E.M.*	C.R.†
Exp. 1. White rats (*Mus norvegicus*)										
0	12	5.7	0.8	0.3	7.6	1.1	0.4	18+	5+	0.4
0.5 in ration	9	6.0	0.7	0.1	8.4	1.5	0.5	21+	5+	0.3
0.2 in drinking water	14	5.6	0.5	0.5	6.9	1.0	0.8	16+	3+	0.8
Exp. 2. Cotton rats (*Sigmondon hispidus hispidus*)										
0	3	10.7	0.5	0	25.7	3.5	0.5	77+	19+	0.3
0.2 in drinking water	3	10.7	0.5		23.3	3.1		69+	13+	

* Standard error of mean.

† Critical ratio—the ratio of the difference between 2 means to the standard error of the difference between the means. Wherever the critical ratio is less than 2.0, the difference between the means is considered to be statistically insignificant; when the critical ratio is between 2.0 and 2.9, the difference is of borderline significance; when the critical ratio is 3.0 or higher, the difference is highly significant. The criss-cross lines in the above table connect sets of data which are being compared for the critical ratio test of significance.

There was no evidence of a toxic effect of the glyceraldehyde additions.

The average caries experiences of the groups of white rats and cotton rats fed racemic glyceric aldehyde in the ration or in the drinking water, and of comparable control groups are presented in Table I. In the white rat, the consumption of a purified caries-producing ration supplemented with 0.5% of dl-glyceric aldehyde in the dimeric form did not influence the average number of carious molars, the average number of carious lesions, nor the average extent of tooth substance affected by the carious processes. Likewise, the consumption of water containing 0.2% dl-glyceric aldehyde as the monomer throughout the experimental period of maintenance on the purified ration did not alter the caries experience of both species.

Discussion. The results of the above experiments and of those reported previously[2] indicate the inability of racemic glyceric aldehyde, as the dimer or as the monomer, to alter the initiation and rate of development of carious lesions in experimental animals maintained under the conditions of these studies. These observations are in contrast to those which indicated the strong ability of glyceric aldehyde to inhibit acid production in incubated saliva. For a number of years, dental investigators in several laboratories have suggested that compounds which were able to inhibit acid production in incubated saliva or in incubated cultures of *Lactobacillus acidophilus* would have a similar inhibitory effect on the initiation and development of tooth decay in experimental animals and human beings. In view of the divergent results between *in vitro* studies and animal experiments with racemic glyceric aldehyde, the above generalization does not appear to be valid. Careful examination of suggestive *in vitro* findings by suitable *in vivo* procedures with caries-susceptible laboratory animals and later with human subjects is necessary to establish the exact value of any compound or treatment for caries prevention. The striking gross and histologic similarity between experimentally produced carious lesions in the Norway rat[4] and in the cotton rat[5] to the naturally occurring lesions in human teeth is indicative of the validity of these two species for the evaluation of *in vitro* inhibitors prior to clinical application.

Summary. The addition of 0.5% of dl-glyceric aldehyde to a caries-producing ration or of 0.2% dl-glyceric acid to the drinking water did not reduce the initiation nor the rate of development of carious lesions in caries-susceptible white rats and cotton rats.

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[4] Sognnaes, R. F., *J. Nutrition*, 1948, **36**, 1.

[5] Shaw, J. H., *J. Nutrition*, 1949, **38**, 275.

Received August 15, 1949. P.S.E.B.M., 1949, **72**.

Symbiotic Growth of Pleuropneumonia-like Organisms with Bacterial Colonies. (17423)

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A symbiotic relationship among bacteria was first described by Grassberger.[1] He observed that in the vicinity of colonies of various bacteria, particularly *Staphylococcus aureus, Hemophilus influenzae* grew as satellite colonies on blood agar. It is now well known that the staphylococci synthesize necessary growth factors required by *Hemophilus influenzae* which are not contained in the medium.

A similar symbiotic relationship has been observed with the pleuro pneumonia-like organisms (PPLO). In the course of our work

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* Difco Laboratories Fellow in Bacteriology.

[1] Grassberger, R., *Z. f. Hyg.*, 1897, **25**, 453.

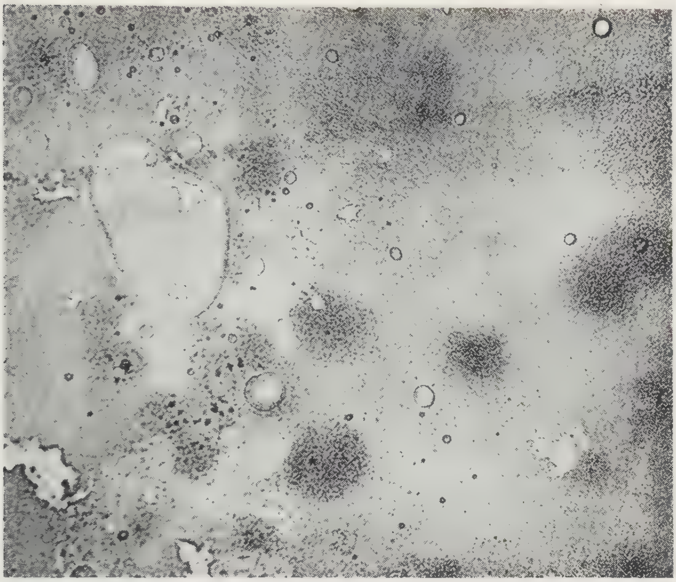

FIG. 1.
PPLO colonies, strain No. 17 from cervical secretion, growing under *Staphylococcus albus.* When the agar block containing the staphylococcal and PPLO colonies was stained by the method of Dienes, the entire bacterial growth took the blue due. After a short time the staphylococcal colonies reduced the dye leaving the intensely stained PPLO colonies as distinct islands. 130 ×.

with these organisms a gram-positive coccus occurred as a contaminant on a beef heart infusion agar plate (without ascitic fluid) which had been inoculated with the PPLO. The PPLO were noticed directly underneath the bacterial colonies. The gram-positive cocci were identified as *Staphylococcus albus.* An attempt was then made to determine whether this phenomenon would occur with other PPLO strains, many of which had been isolated from clinical sources. The PPLO were never observed to grow on heart infusion agar medium containing Bacto-beef heart 5.0%, Bacto-peptone 1.0%, NaCl 0.5%, and agar 1.5% unless ascitic fluid or blood serum was added. The entire areas of beef heart infusion agar plates without ascitic fluid were inoculated with the different strains of PPLO by streaking the surfaces with agar blocks containing the L type colonies. Inoculation of the staphylococci was accomplished by making one streak across each of the plates after the inoculation with PPLO. After five days incubation aerobically at 37°C nine of fourteen strains from human sources and one strain from a rat grew underneath the staphylococcal colonies. In the isolated areas where there were no staphylococcal colonies the PPLO did not grow. This experiment was repeated using a strain of *Proteus vulgaris.* All 15 strains of the PPLO grew and, as was the case with the staphylococcus, only underneath the bacterial colonies. In neither case were any structures similar to the L type colonies observed in growths of the *S. albus* and *P. vulgaris* on the medium which was not inoculated with PPLO.

The colonies of PPLO were detected by direct microscopic examination of the plates at a magnification of approximately 100X. Their identity was verified by cutting out blocks of agar and staining the growths by the method of Dienes. Photographs of two stained preparations are reproduced as Fig. 1 and 2.

This report is made for possible usefulness to those interested in cultivating the PPLO from clinical materials or to those interested in the metabolism of this little-understood

FIG. 2.
PPLO colonies, strain No. 36 from prostatic secretion, growing under *Proteus vulgaris.* When an agar block containing the growth of the two types of organisms was stained as described for Fig. 1 the PPLO colonies, with this strain, appeared as small darkly stained areas.

group of microorganisms.

Summary. Nine of 14 strains of PPLO of human origin and one strain of rat origin were observed to grow on beef heart infusion agar without ascitic fluid or serum only in symbiosis with *Staphylococcus albus* and all 15 strains grew in symbiosis with *Proteus vulgaris.*

Received August 16, 1949. P.S.E.B.M., 1949, **72**.

A Simple, Quantitative Method for Titration of Trypsin and Trypsin-Inhibitors.* (17424)

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In recent years the interest of an increasing number of investigators in the mechanism of fibrinolysis[1] and related phenomena, and especially in the role played by the activators and inhibitors of natural plasma proteases in the complicated process of blood coagulation,[2-5] has led to improved procedures for

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* This investigation was supported in part by a grant from the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service.

[1] Christensen, L. R., *J. Gen. Physiol.*, 1945, **28**, 363.

[2] Ferguson, J. H., *Science*, 1943, **97**, 139.

[3] Tagnon, H. J., *J. Lab. and Clin. Med.*, 1942-43, **27**, 1119.

studying tryptase-antitryptase systems. The fibrinogenolytic method for measuring blood proteases and protease inhibitors, as first suggested by Ferguson,[6] or in somewhat modified form,[7–9] has proven especially useful. These procedures, however, leave something to be desired for routine tests on a large scale.

The need for a truly simple, quantitative technic for accurate titration of the changes in the protease inhibitor titers of body fluids associated with various physiologic and pathologic states, and particularly in connection with allergic phenomena, has been obvious for a long time. The concept that hypersensitivity reactions are caused primarily by toxic products formed, or released, when the natural proteases are suddenly activated following antigen-antibody union, was originally expressed many years ago, as was also the correlated idea that allergic reactions are controlled by the action of protease inhibitors.[10-12] Until recently very little precise data in direct support of this view has been published, however. The importance of supplying such data on an adequate scale led one of us some years ago to attempt development of a quantitative procedure for antitrypsin titration especially suitable for use by immunologists.[13–15] The method was based upon the fact that when properly prepared photographic film is brought into contact with solutions of active trypsin the gelatin is digested, releasing the silver and clearing the film to a degree proportional to the activity of the trypsin. When digestion is complete the originally opaque, black film becomes completely transparent.

The present paper outlines the vastly improved technic for the performance of this film-gelatin-digestion method developed during the past two years.

Reagents. 1. *Stock buffer solution pH 7.5* is made by mixing 1.3 parts of M/20 sodium borate (19.1 grams $Na_2B_4O_7$:10 H_2O per liter of distilled water) with 8.7 parts of M/5 boric acid-salt solution (12.4 grams H_3BO_3 and 2.9 g NaCl per liter of distilled water). This reagent is used for all dilutions of trypsin and of sera or other fluids under test.

2. *M/5 sodium borate solution* (76.3 grams Na_2B_4:10 H_2O per liter of distilled water) is kept in a sealed bottle in a 37°C incubator.

3. *Stock crystalline trypsin solution* (*CT*) is prepared by dissolving 0.3 g of commercial crystalline trypsin[†] in 100 ml of a glycerin-buffer mixture consisting of equal volumes of stock buffer solution and C.P. glycerine; the pH is about 4.4. It is stored in a sterile, chemically clean, screw-top bottle at 4-10°C. Freshly-made solutions are allowed to stand undisturbed in the refrigerator 24 hours before use. (A precipitate appearing at this time is removed by filtering through paper.) The effect of the glycerine and the low pH is to stabilize the enzyme[16] so that the proteolytic activity of the solution remains unchanged for many months.

4. *Working trypsin solution* (*WT*) is made by mixing one part of stock trypsin solution with two parts of the M/5 sodium borate solution (Reagent 2). This reduces the concentration of trypsin to 0.1% (1 mg/ml) and the pH is brought to 6.8. It is practicable to make up this reagent in a volume of 100 ml or more. Freshly made solutions are allowed to stand undisturbed in the refrigerator for 24 hours before use. When stored in

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[4] Glazko, A. S., *J. Clin. Invest.*, 1947, **26**, 364.

[5] Milstone, J. H., *Science*, 1947, **106**, 546.

[6] Ferguson, J. H., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 243.

[7] Ferguson, J. H., Travis, B. L., and Gerheim, E. B., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 285.

[8] Christensen, L. R., *J. Clin. Invest.*, 1949, **28**, 163.

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[10] Bronfenbrenner, J., PROC. SOC. EXP. BIOL. AND MED., 1915-16, **13**, 42.

[11] Bronfenbrenner, J., and Schlesinger, M. J., *J. Immunol.*, 1918, **3**, 321.

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[13] Burdon, K. L., and Lafferty, C., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 787.

[14] Burdon, K. L., *J. Bact.*, 1941, **41**, 62.

[15] Burdon, K. L., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 24.

[†] Worthington Biochemical Laboratory, Freehold, N. J., or Armour Laboratories, Chicago, Ill.

[16] Burdon, K. L., *Science*, 1941, **93**, 91.

TABLE I.
Scheme for Dilution of Working Trypsin Solution.

Dilution No.	Working trypsin, ml*	Buffer, ml*	Trypsin conc., mg/ml		Tryptic units (TU)
			In dilution	Final†	
1	.4	7.6	.05	.025	.5
2	.5	7.5	.063	.031	.62
3	.6	7.4	.075	.037	.75
4	.7	7.3	.087	.044	.87
5	.8	7.2	.1	.05	1.0
6	.9	7.1	.113	.056	1.12
7	1.0	7.0	.125	.062	1.25
8	1.1	6.9	.138	.069	1.37
9	1.2	6.8	.15	.075	1.5
10	1.3	6.7	.163	.081	1.62
11	1.4	6.6	.175	.087	1.75
12	1.5	6.5	.188	.094	1.87
13	1.6	6.4	.2	.1	2.0
14	1.7	6.3	.213	.106	2.12
15	1.8	6.2	.225	.112	2.25
16	1.9	6.1	.238	.119	2.37
17	2.0	6.0	.25	.125	2.5
18	2.1	5.9	.263	.131	2.62
19	2.2	5.8	.275	.137	2.75
20	2.3	5.7	.288	.144	2.87
21	2.4	5.6	.3	.15	3.0

* The amounts required to make up a total volume of 8.0 ml are shown because this is often a convenient quantity for routine tests.

† After mixture with equal volume of buffer, or diluted specimen, as in the test proper.

sterile, chemically clean screw-top bottles at 4-10°C the tryptic activity remains constant for at least 6 months.

5. *The fixing solution* consists of 95% ethyl alcohol to which 10% by volume of 28% glacial acetic acid is added.

6. *The filmstrips* are cut from the special film prepared to our specifications by the Eastman Kodak Company.‡ The film is a duplitized, 35 mm ciné-film, flashed on both sides to a density of 1.0, so that it has a total density of about 2.0. Both the density and the hardness of the emulsion are uniform throughout. The reels of stock film are kept at all times in tightly sealed cans in the refrigerator. A supply of strips is made, when needed, by cutting the film crosswise into pieces ¼ inch wide with a small paper cutter. The width of the strips need not be exact. About ¼ inch from the end each strip is bent upward at nearly a right angle to make it easier to grasp with forceps. In handling the film it is important to avoid finger marks, and exposure to heat or dust. The newly-cut strips are transferred at once to clean, screw-top jars or tubes, and thus kept carefully sealed in the refrigerator until actually used in a test.

I. *Trypsin Dilution Method.* 1. *Initial dilution of the serum or other fluid to be tested.* The majority of specimens of normal human, rabbit, or guinea pig sera will give end-points in a convenient range when they are diluted originally with stock buffer 1:50. For some high-titer sera, or other fluids, it may be found desirable to use a different initial dilution (*e.g.* 1:100). In any series of comparative tests all specimens should be titrated at the same initial dilution.

2. *Dilutions of the working trypsin solution.* For the *trypsin control* tubes in routine tests it is necessary to prepare only dilutions 3, 4, and 5 (Table I). For the *test mixtures* a series of six or eight additional dilutions are usually made through a range likely to give end-points in the presence of the serum or

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‡ We wish to express our sincere appreciation to Dr. Burt H. Carroll of the Research Laboratories, and Mr. W. F. Swann of the Industrial Photographic Sales Division for their generous cooperation in the development and production of this essential material.

other fluid under test.

All dilutions are made with the stock buffer solution in chemically clean glassware, using plugged, 1 ml serological pipettes. The trypsin control dilutions have a pH of 7.5 and succeeding tubes in any part of the test series differ in hydrogen ion concentration by only about 0.01 pH.

These dilutions gradually lose activity at room temperatures, especially at temperatures over 85° F., but the same set of solutions may be used throughout a working day if the tubes are kept in a pan of ice water, or if they are placed in the refrigerator except when actually being used.§

3. *Preliminary adjustment of the incubation time.* A new working trypsin solution is first titrated in dilutions 1 to 7 under the standardized conditions of the test proper, as outlined below, except that the digestion incubation time in the 40°C water bath is varied in successive runs until the time required to give the end-point regularly with dilution No. 5 (Table I) is determined. The tube made up with dilution No. 5, plus an equal volume of stock buffer, will thus have, as intended, just *one unit of tryptic activity, i.e.* it will contain the amount of WT just sufficient to digest all the gelatin (clear the film completely) in a certain time at 40°C. It will be seen (Table I) that the final concentration of crystalline trypsin in this end-point tube is 0.05 mg/ml.‖ The time required with the present lot of film used by the writers is 6½ minutes.

First tests are commonly run at times differing by half-minute time intervals, then by quarter-minutes and finally by one-eighth minutes. The incubation time, once chosen on the basis of these tests, is kept constant throughout all future tests with that lot of working trypsin solution, and indeed, rarely requires any adjustment so long as the same *stock* trypsin solution is used. Thus, strictly comparable titrations can be obtained in separate runs carried out weeks or months apart.

4. *The test proper. a.* Ordinary serological tubes (12 x 75 mm) are arranged in a water bath rack. Using a separate, plugged 1.0 ml pipette for each dilution, 0.4 ml of dilutions 3, 4, and 5 of the working trypsin solution is introduced into a series of 3 tubes that are to serve as trypsin controls, and 0.4 ml of other appropriate dilutions is placed in a further series of 6 or 8 additional tubes. Now, 0.4 ml of the stock buffer solution is added to each of the control tubes, and 0.4 ml of the diluted serum, or other fluid under test, to each of the remaining tubes. The rack is now shaken, as in a Kahn test, for one-half minute, then placed in a 40°C water bath¶ with a timer set for 10 minutes.

b. The filmstrips are now obtained from their jar in the refrigerator and arranged in parallel rows on some convenient holder (such as a small slide-box lid) so that they can be more readily picked up one by one with forceps.

c. At the end of the warming period the interval timer is carefully reset for the test incubation time, *e.g.* 6½ minutes. The timer is started and the introduction of the filmstrips into the test tubes (still in the water bath) is begun immediately. The strips are added one by one in a definite order, and following a rhythmic time pattern which must be perfected by practice.

d. The fixing solution and equipment are now obtained from the refrigerator. Some of the solution is poured into a 600 ml beaker and heavy-walled capillary pipettes with large rubber bulbs attached are filled with it.

e. When the timer rings the rack is removed immediately from the water bath, and then, without delay, the cold fixing solution is added to each tube in the same order, and following the same time pattern in which the filmstrips were originally introduced. The fixing solu-

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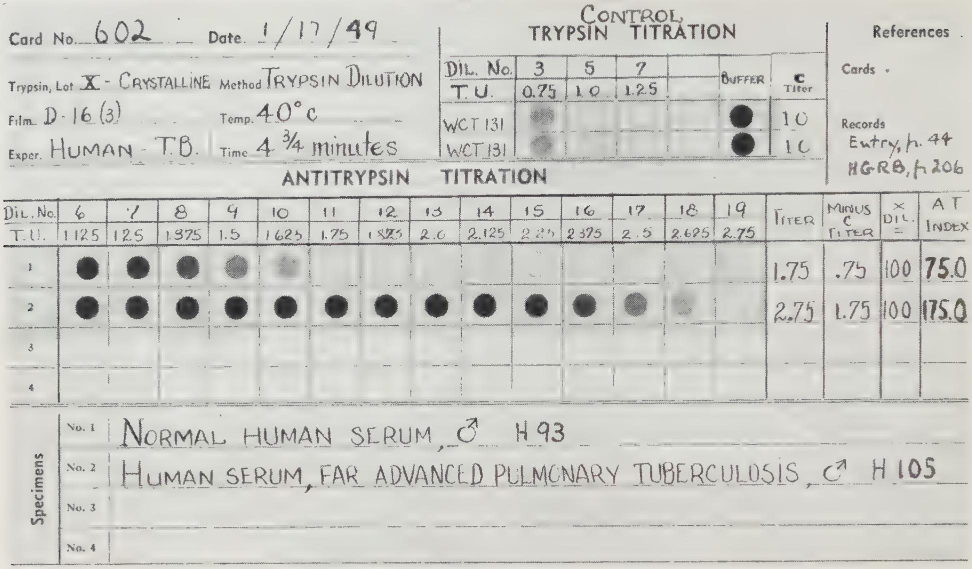
§ The stability of the reagents and the effects of various other factors upon the validity of the tests have been experimentally studied, and findings will be reported in a separate communication.

‖ The fact that the powdered commercial crystalline trypsin contains approximately 50% $MgSO_4$ is disregarded.

¶ Best results have been obtained by the use of an Army-type electric water bath (Chicago Surgical and Electrical Co.) with separate gable cover over each rack.



Card No. 602 Date 1/17/49

Trypsin, Lot X - CRYSTALLINE Method TRYPSIN DILUTION

Film D-16 (3) Temp. 40° c

Exper. HUMAN - T.B. Time 4 ¾ minutes

CONTROL TRYPSIN TITRATION

Dil. No.	3	5	7		Buffer	C Titer
T.U.	0.75	1.0	1.25			
WCT 131	●				●	1.0
WCT 131	●				●	1.0

References

Cards

Records
Entry, p. 44
HGRB, p. 206

ANTITRYPSIN TITRATION

Dil. No.	6	7	8	9	10	11	12	13	14	15	16	17	18	19	Titer	Minus C Titer	× Dil. =	AT Index
T.U.	1.125	1.25	1.375	1.5	1.625	1.75	1.875	2.0	2.125	2.25	2.375	2.5	2.625	2.75				
1	●	●	●	●	●										1.75	.75	100	75.0
2	●	●	●	●	●	●	●	●	●	●	●	●	●		2.75	1.75	100	175.0
3																		
4																		

Specimens
No. 1 NORMAL HUMAN SERUM, ♂ H 93
No. 2 HUMAN SERUM, FAR ADVANCED PULMONARY TUBERCULOSIS, ♂ H 105
No. 3
No. 4

FIG. 1.

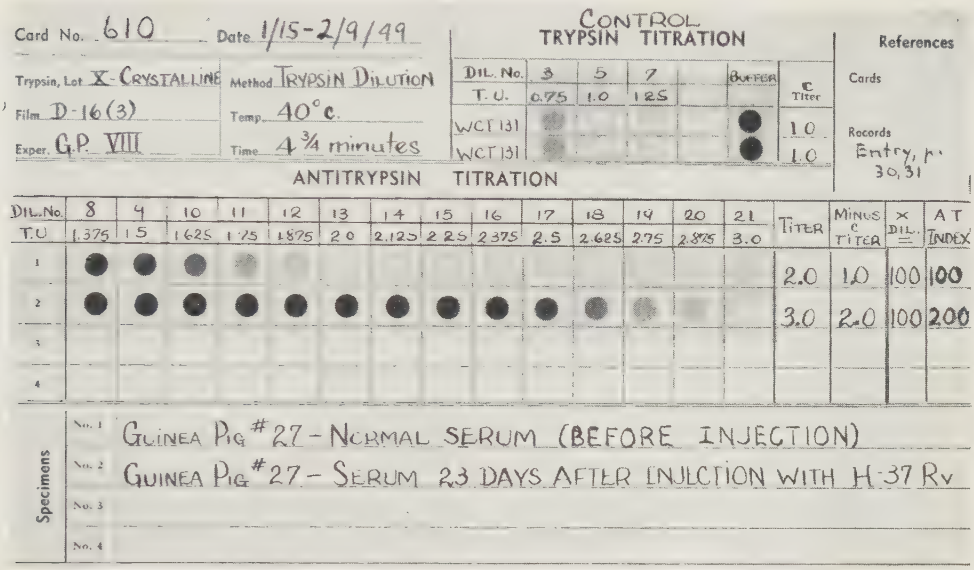
tion is added with sufficient force to cause the strips to float upward somewhat, thus bringing the fixative into contact with the film throughout its length. The rack is now placed in the refrigerator for 5 minutes to assure the full hardening of any of the gelatin emulsion remaining undigested.

f. The filmstrips are now removed from the tubes with forceps, rinsed briefly in fresh, cold fixing solution, and touched, at the edges only, to blotting paper. They are then inverted and set upright in a bed of plasticine so that they will drain and dry uniformly.

5. *Reading of the test; preparation of the permanent record card.* By simple inspection of the dried strips when held to the light, the end-points (the first completely cleared films) in the trypsin control, and in the test series, are determined and recorded. From each strip a disc 5 mm in diameter is then cut from the digested portion with an ordinary paper punch. These discs are mounted, with the aid of Scotch tape, upon a white card which bears the desired descriptive data. Thus a permanent record card for filing may be made of every test. (Fig. 1, 2).

6. *Expression of the results.* The *titer of proteolytic activity* may be expressed most conveniently in terms of tryptic units (TU). By preliminary adjustment of the incubation time, as described above, the titer in the trypsin control tubes (in all properly performed tests) is always *1.0 TU*. The *difference* between this titer and that obtained in the test series of tubes in the presence of a specimen of serum or other inhibiting material represents the antitryptic power of that sample. A final figure to represent the *antitryptic index* is obtained by multiplying this difference by the final dilution (*i.e.* double the initial dilution) of the specimen. For example, in the presence of specimen H-93 (Fig. 1) the titer is 1.75 TU, a difference of 0.75 over the control titer of 1.0 TU. This difference, times the final dilution, gives the antitryptic index: 0.75 x 100 = 75.0.

The antiprotease action may also be expressed more directly in terms of the amount of trypsin inhibited. Thus specimen H-93, in final dilution 1:100, (Fig. 1) may be said to have prevented the activity of 0.0375 mg/ml of trypsin, the difference between the 0.0875 mg/ml in the first tube showing 100% digestion (No. 11) and the 0.05 mg/ml present in the control end-point tube (No. 5). Each milliliter of *undiluted* H-93 has the apparent capacity to inhibit the action of 3.75 mg/ml crystalline trypsin. The correspond-



Card No. 610 Date 1/15-2/9/49

Trypsin, Lot X-CRYSTALLINE Method TRYPSIN DILUTION

Film D-16(3) Temp. 40°c.

Exper. G.P. VIII Time 4¾ minutes

CONTROL
TRYPSIN TITRATION

DIL. No.	3	5	7		BUFFER	c Titer
T.U.	0.75	1.0	1.25			
WCT 131	◌				●	1.0
WCT 131	◌				●	1.0

References

Cards

Records
Entry, p.
30, 31

ANTITRYPSIN TITRATION

DIL. No.	8	9	10	11	12	13	14	15	16	17	18	19	20	21	TITER	MINUS c TITER	× DIL. =	AT INDEX
T.U.	1.375	1.5	1.625	1.75	1.875	2.0	2.125	2.25	2.375	2.5	2.625	2.75	2.875	3.0				
1	●	●	●	◌	◌										2.0	1.0	100	100
2	●	●	●	●	●	●	●	●	●	●	●	◌	◌		3.0	2.0	100	200
3																		
4																		

Specimens
No. 1 GUINEA PIG #27 - NORMAL SERUM (BEFORE INJECTION)
No. 2 GUINEA PIG #27 - SERUM 23 DAYS AFTER INJECTION WITH H-37 Rv
No. 3
No. 4

Fig. 2.

ing figures for undiluted specimens H-105, G.P. No. 27 normal, and tuberculous (Fig. 2) are 8.75, 5.0, and 10 mg/ml respectively.

Again, the antitryptic effect may be compared with that exhibited by a chemically purified material, such as commercial crystalline soybean trypsin inhibitor (CSBI).[†] We have found that one TU (*i.e.* a working trypsin solution which in final dilution contains 0.05 mg/ml CT) is *completely* inhibited by only 0.0167 mg/ml CSBI, or a proportion of inhibitor to trypsin of 1:3, and that *partial* inhibition continues so long as the final concentration of CSBI is not less than one part to 5.6 parts of trypsin. In the presence of 6 parts of trypsin the film is completely digested. Each milliliter of *undiluted* specimen H-93 (Fig. 1) may thus be calculated to have shown an inhibiting power equivalent to that of 0.625 mg/ml of CSBI,—0.0875 mg/ml (the amount of trypsin present in the end-point tube) minus 0.05, divided by 6 = 0.00625 mg/ml x the final serum dilution (1:100) = 0.625 mg/ml. For H-105, G.P. No. 27 normal, and tuberculous, the figures are 1.46, 0.833 and 1.66 mg/ml respectively.

Finally, advantage may be taken of the fact that the relative amount of clearing of the filmstrips in the several tubes just preceding the end-point tube is as reproducible as the end-point itself, and these strips may be read in terms of *percentage of digestion,* thus furnishing in some instances a distinction between two specimens, both of which have shown the same end-points. In other words, antitryptic indexes based on end-points do not always express all the degrees of tryptase-inhibiting activity possessed by different sera, even when the differences between the succeeding trypsin dilutions used is as small as is practicable, *i.e.* only 0.125 tryptic units, as in Table I. The estimate of the percentage digestion is made by close comparison under uniform illumination of the film discs in question with a standard set of discs showing 20 graduations from zero to 100% digestion. These readings are valuable in reinforcing the information obtained by comparison of end-points, and could be utilized for further statistical analysis of the findings.

II. *Specimen Dilution Procedure.* The purpose in this form of the test is to determine the highest dilution of the serum or other fluid under test that will inhibit in any degree the complete clearing of the film in the presence of one TU of trypsin at the standard time and temperature.

1. *Preliminary steps.* A sufficient volume

of the working trypsin solution is made up in dilution No. 5 (Table I) to supply the 0.4 ml required for a test with each of 8 or 10 specimen dilutions, plus a control. Small amounts of dilutions 3, 4, and 5 are also made up separately to be run as independent trypsin controls. The specimen is diluted with stock buffer by the two-fold dilution procedure generally used in agglutination titrations, or by some other convenient scheme. The great majority of serum specimens from human beings, guinea pigs, or rabbits will give titers within the range 1:64 to 1:2500.

2. *The test proper.* To 0.4 ml of each of the specimen dilutions is added 0.4 ml of WT, dilution No. 5. Control tubes containing 0.4 ml of this identical trypsin solution + 0.4 ml of buffer, as well as the separately-made trypsin controls, are set up. The remaining steps of the test are now carried out as in the Trypsin Dilution Method outlined above.

3. *Expression of the results.* The titer of antitryptic activity here exhibited is ordinarily stated in terms of the highest final dilution of the specimen that inhibits in any degree the clearing of the film. The controls must show that the trypsin solution used in all the test mixtures contained, as intended, one TU. The results may also be stated in terms of the amount of trypsin inhibited, or they may be interpreted in comparison with the action of known amounts of crystalline soy bean inhibitor. A reading of the percentage of digestion at the titer dilution may also be made, differing degrees of inhibiting action may thus be revealed in different sera having the same dilution titer.

Use of crude trypsin. The film-gelatin digestion method was originally developed before crystalline trypsin preparations became available commercially, and the procedures outlined above may still be used without essential change for tests with extracts of powdered crude trypsins. The stock trypsin solutions are made by weighing out 6 g of Fairchild's trypsin,** or 3 g of other commercial preparations†† and placing the powder in a mortar. One hundred milliliters of glycerine-buffer solution (equal parts of C.P. glycerine and stock buffer) are added gradually, stirring in a small amount at first to make a paste. The mixture is transferred to a stoppered flask and allowed to stand at room temperature for about 2 hours, with occasional stirring, then overnight in the refrigerator. A crystal clear extract (pH about 5.8) is finally obtained by filtering through S and S No. 588 paper, or preferably through a Seitz filter after adding one percent of filter-aid.‡‡ These glycerin-buffer extracts are stable over long periods of time[16] when stored in a chemically-clean, screw-top bottle in the refrigerator.

Comparative activity of crude and crystalline trypsin solutions; differences in reaction with trypsin inhibitors. Crystalline trypsin solutions were found to have almost exactly the same activity in the filmstrip tests as the extracts obtained from *10 times* the weight of the currently available crude powdered trypsins. Hence, with the crude trypsin extracts made as described above end-points are obtained in a similar range of dilutions as with crystalline trypsin, and the same scheme of tryptic units may be used to express the titers obtained.

However, the amount of trypsin *inhibitor* revealed in serum and other materials is much greater in tests with crystalline trypsin than in parallel titrations with the less pure enzyme preparations. Thus, the antitryptic indexes of various sera tested with the newer powdered trypsins were found to be about twice those obtained in titrations with the older, relatively impure, Fairchild's product. Crystalline trypsin again regularly reveals more than twice as much inhibitor as is shown in comparable tests with any of the currently available crude trypsins. Thus, the average antitryptic index of 23 human sera, (initial dilution 1:50), tested in parallel, was 51.6 with one of the powdered trypsins and 114.1 with crystalline trypsin. The difference,

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** Fairchild Bros. and Foster, N. Y. ("Trypsin 1:70"). This material is no longer being manufactured.

†† ("Trypsin 1:300"), Nutritional Biochemicals Corporation, Cleveland, Ohio, or "Pancreatic Trypsin," Bios Laboratories, N. Y.

‡‡ Hyflo Super-Cel, Fisher Scientific Co., Pittsburgh, Pa.

moreover, is greatest when the inhibitor content of the specimen is especially high. Evidently the impurities in crude trypsin solutions prevent effective contact or combination of a considerable proportion of the antitryptic substances with the active trypsin molecules.

Despite these limitations, antitrypsin titrations with crude trypsin extracts have revealed data of apparent significance in support of the autodigestion theory of allergy.[13,17] For critical studies, however, only tests with crystalline trypsin can now be recommended.

Comment. It is important to realize that all of the procedures outlined above require meticulous attention to detail, and can only be carried out successfully by a conscientious technician, since the slightest deviation from the required technic shows up inexorably. Nevertheless, the tests are readily adaptable to routine use and an average of 20 separate specimens a day may be titrated by a single, practiced worker.

The method makes it feasible to add to the data ordinarily obtained in experimental and clinical studies of allergy precise information on the protease-inhibiting power of the sera of patients and experimental animals, or of the reagents used. The titrations should aid materially in appraising the significance of the previously-neglected antitryptic effect *in vivo* or *in vitro* of the materials and manipulations employed in any experiment.

The basic film-gelatin-digestion technic should prove readily adaptable also for the titration of proteases other than trypsin, for experiments on the kinetics of these enzyme reactions and for the study of important physiologic and pathologic phenomena, other than hypersensitivity, in which natural proteases, their activators and inhibitors, appear to play so vital a part.

Summary. A technically simple, rapid method for the quantitative titration of trypsin or similar proteolytic enzymes, and of protease inhibitors, suitable for critical tests on a large scale is described. The procedure utilizes technics familiar to serologists, rather than to skilled biochemists only, and gives definitive, reproducible results that can be expressed in simple figures. A feature is the provision for the making of a permanent record of each titration. Since the reagents are stable, and readily standardized, comparable findings can be obtained in separate experiments conducted in the same or in different laboratories at any time.

The assistance of Mrs. David A. Cook with records and statistics is gratefully acknowledged.

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[17] Burdon, K. L., and Mudd, R. P., Proc. Soc. Exp. Biol. and Med., 1949, in press.

Received August 15, 1949. P.S.E.B.M., 1949, **72**.

Quantitative Paper Chromatography: A Simplified Procedure. (17425)

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The now classical paper chromatographic method of Consden, Gordon and Martin[1] has been applied to the separation of many types of organic and inorganic substances. Although the method as originally described was only qualitative or roughly quantitative (± 50%), a number of quantitative adaptations have been published. These adaptations may be classified into several general categories: (A) the paper chromatogram is cut so that each substance separated may be extracted with the appropriate solvent and then the concentration of the material in the extract is determined by conventional colorimetry or other means (*cf.*[2]); (B) the materials on the chromatogram are revealed by their own color, by reaction with specific reagents to produce a colored compound, by their microbiological effects when the paper is placed on a suitable test medium, or by radioactivity. In such instances, it has been found by Fisher, Parsons and Morrison[3] that the area on the paper occupied by each substance is proportional to the logarithm of its concentration. This procedure is very satisfactory when the boundaries of the spots are sharply delineated, but cannot be applied with accuracy unless this is so or when the substances are not distinctly separated from each other. (C) In order to overcome these disadvantages, a method has been described[4,5] in which the color density is determined continuously or in overlapping increments with an electronic densitometer along the entire length of a one dimensional chromatogram strip. The color densities, so obtained, are plotted on graph paper, a series of peaks are obtained, and the concentration of each substance or substances is calculated from the area under each density peak. This procedure is very satisfactory for the determination of substances which are well or partly separated from each other, but is exceedingly tedious unless automatic equipment is available. Furthermore, as there is no known single solvent which permits satisfactory separation of all or almost all the amino acids present in a protein hydrolysate, preliminary separation by adsorption, ion exchange or paper chromatography must be resorted to in many instances.[4,6] (D) One of the advantages of the paper chromatogram technic of Consden, Gordon and Martin[1] over the older capillary analysis method of Schoenbein and Goppelsroeder (*cf.*[2]) is the two-dimensional chromatogram. The method often gives even greater resolution than can be obtained by preliminary separation by adsorption, ion exchange or large scale paper chromatography followed by one-dimensional strip chromatography and is much easier to conduct. It has been reported[4] that the concentration of a colored substance on a two-dimensional paper chromatogram can be estimated from the product of the greatest color density times the area of the spot.

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[1] Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, 1944, **38**, 224.

The data to be given in this paper show that the concentration of colored substances separated by paper chromatography may be estimated directly on the chromatograms, with reasonable accuracy, either from the product of the area times the color density or more simply from the color density alone. The examples given are for the estimation of amino acids. However, equal success has been achieved with non-volatile amines and it appears that the same principle would apply to other colored substances on paper chromatograms.

1.) Determination of Histidine and Tyrosine in Protein Hydrolysates (*cf.*[7]) *Experimental.* Histidine and tyrosine are separated by paper chromatography employing S. & S. 598 paper with a solvent mixture consisting of N-butanol 100 parts, glacial acetic acid 10 parts, saturated with water. The chromatograms are run in air tight glass chambers (*cf.*[8]) for 3 hours. The paper is then removed, dried in air, and sprayed with freshly diazotized sulfanilamide dissolved in N-butanol. The chromatogram is again dried in air for exactly 5 minutes and then it is sprayed with saturated Na_2CO_3 solution. The paper is dried in air and the quantities of histidine and tyrosine are determined from the product of the area of the spot times its maximum color density.[4]

Results. A few typical results on standard solutions are given in Table I. In order to ascertain the value of this procedure on protein hydrolysates, histidine and tyrosine analyses were carried out on 3 proteins, the histi-

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[2] Schoenbein, C. F., *Verh. d. Naturforsch. Ges. Basel,* 1861, **3**, 249; Rheinboldt, H. in Houben, *J. Die Methoden der Organischen Chemie,* G. Thieme, Leipsig, 1925, **1**, 291.

[3] Fisher, R. B., Parsons, D. S., and Morrison, G. A., *Nature,* 1948, **161**, 764.

[4] Block, R. J., *Science,* 1948, **108**, 608.

[5] Bull, H. B., Hahn, J. W., and Baptist, V. H., *J. Am. Chem. Soc.,* 1949, **71**, 550.

[6] Block, R. J., and Bolling, D., The Amino Acid Composition of Proteins and Foods, C. C. Thomas, Springfield, Ill., 2nd ed., 1950.

[7] Bolling, D., Sober, A. H., and Block, R. J., *Fed. Proc.,* 1949, **8**, 185.

[8] Nachod, F. C., Ion Exchange: Theory and Application, Academic Press, New York, 1949, p. 308.

TABLE I.
Standard Curves for Tyrosine and Histidine.

	mg	A	D	A × D	Factor
Tyrosine	.0090	18.5	.22	4.07	.0022
	.0090	26.5	.14	3.71	.0024
	.0090	16.5	.24	3.96	.0023
	.0180	27.0	.28	7.56	.0024
	.0180	40.5	.20	8.10	.0022
	.0180	24.5	.30	7.35	.0024
	.0180	37.5	.22	8.25	.0022
	.0180	29.5	.25	7.38	.0024
	.0270	53.2	.20	10.64	.0025
	.0270	50.0	.24	12.00	.0023
	.0270	28.0	.38	10.64	.0025
					.0024
Histidine	.0078	12.5	.32	4.00	.0020
	.0078	12.0	.34	4.08	.0019
	.0156	18.5	.43	7.96	.0020
	.0156	16.5	.41	6.77	.0023
	.0156	20.5	.40	8.20	.0019
	.0156	22.0	.38	8.36	.0019
	.0156	16.5	.43	7.10	.0022
	.0156	26.5	.26	6.89	.0023
	.0234	30.0	.35	10.50	.0022
	.0234	21.3	.56	11.93	.0020
					.0021

A—Area. D—Density. Factor—mg/A × D.

TABLE II.
Tyrosine and Histidine in Proteins.
(Calculated to 16.0 g of nitrogen).

Protein	mg used	Tyrosine, g	Histidine, g
Hemoglobin	.04-.32	3.0	8.2
Casein	.10-.60	5.9	2.9
Zein	.15-.90	5.1	1.8

dine and tyrosine ratios of which differ widely. These results are summarized in Table II. The values obtained agree with the best in the literature (*cf.*[6]).

2.) Estimation of Amino Acids on Two-Dimensional Paper Chromatograms. During investigations on one-dimensional chromatograms,[4] it was observed that the areas obtained from curves made from the maximum and minimum densities were almost as satisfactory as those prepared from scanning the entire curve. This led to an investigation of the relationship between the maximum color density of the spots on a two-dimensional chromatogram and the concentrations. The following procedure can be used to estimate 14 amino acids on approximately 0.3 mg of hydrolysate N of a protein with the usual type of amino acid pattern, with an average error of less than 10%. Only 5 hours total working time are required. The elimination of the need for determining the area of the spot[4] is an obvious advantage.

Experimental. The protein or peptide is hydrolyzed with HCl or H_2SO_4 and the excess mineral acid is removed in the usual fashion. The solution of amino acids is then diluted so that it contains approximately 1.5 to 10.0 millimols of each amino acid per ml. Isopropanol (to 10%) is used as the preservative. 0.005 or 0.01 ml aliquots of the hydrolysate are applied to the paper and the amino acids are then separated by two-dimensional chromatography on S. & S. No. 596, Whatman No. 4, or other suitable paper. Water-saturated phenol (in an atmosphere of 0.3% of NH_3, moistened NaCN and coal gas) is the first solvent. The chromatograms are then dried in the hood with a blast of warm air until most of the solvent has been removed. Then the sheets are dried before an electric fan over night, 2.5 cm of the leading edge of the phenol run are cut off the paper in order to eliminate the discolored front. The chrom-

TABLE III.
Color Ratios and Amino Acids in Casein.

Amino acid	Standard color ratio	Corrected experimental color ratios (molecular ratio)	Amino acids in casein millimols per 1.60 mg of N	
			Found	Calculated[6]
Arginine	.92	6.4	2.4	2.4
Histidine	1.12	5.1	1.9	2.1
Lysine	.45	14.1	5.4	5.8
Tyrosine	.98	10.0	3.8	3.8
Phenylalanine	.99	9.7	3.6	3.1
Methionine	1.13	6.1	2.3	2.3
Serine	.90	13.2	5.0	5.6*
Threonine	1.02	10.2	3.8	4.0
Leucines	1.08/2	35.8	13.5	12.8
Valine	1.02	15.1	5.7	6.3
Glycine	.89	8.3	3.1	2.8-3.7
Alanine	1.26	9.8	3.7	3.7
Glutamic acid	.72	40.4	15.3	16.1
Aspartic acid	1.33	11.4	4.3	5.6
Proline			++	
Hydroxyproline			—	

* Uncorrected for hydrolytic losses.

atogram is then developed at right angles to the phenol run with 2,-6 lutedine 55 parts, 95% ethanol 20 parts and water 25 parts by volume in an atmosphere of diethylamine and moistened NaCN. At the completion of the run, the chromatograms are dried in the hood by a blast of warm air and the amino acids are revealed by spraying with 0.1 or 0.2% of ninhydrin. The color is brought out by heating the chromatograms. After the color has developed, the maximum color density of each spot is measured with an electronic densitometer (*cf.*[4,5]) using a 5 x 5 mm or a 15.6 mm^2 aperture disc.

Calculation. Mixtures of all amino acids in a protein hydrolysate, with the exception of cystine, tryptophan, proline and hydroxyproline, are prepared so as to contain the following quantities of *each* amino acid per ml. : 1.25, 2.50, 5.00 and 10.00 millimols. Twenty-five or more chromatograms are carried out on each of the above standards. The maximum color density of each amino acid on the chromatograms is then determined. The "mean color density", that is the arithmetic mean of the maximum color densities of *all* the spots on each of the 4 standard concentrations, is then calculated. Similarly, the arithmetic means of the color densities given by each individual amino acid on the 4 standard chromatograms is computed. The latter divided by the former value is the "standard color ratio" of the individual amino acids. "Color ratios" then are calculated in the same manner for all amino acids which are seen on two dimensional chromatograms of acid hydrolysates. These values are called "experimental color ratios." The "standard color ratios" vary less than 10% from run to run over a period of several months and over a range of 1.25 to 10.00 millimols of amino acid per ml. The average "standard color ratios" on S. & S. No. 596 paper are given in Table III.

The "experimental color ratios" of amino acids in the unknown (acid hydrolysate) are determined as above and the values found for each amino acid are then divided by the appropriate "standard color ratio." This gives the "corrected experimental color ratio" of each amino acid on the chromatograms (Table III, 3rd column). The proportion of the "corrected experimental color ratios" to each other gives the molar ratios of each amino acid to the other on the chromatograms. The approximate quantities* of the amino acids on the chromatograms are then readily determined if the concentration of one, or better 2 or 3, of the amino acids are known from independent chromatographic or other methods.

In those cases where one or more amino

acids are absent or present in comparatively small amounts in the hydrolysate under analysis, the addition of known quantities of this amino acid to the hydrolysate will serve as an internal standard.

When 25 replicate chromatograms are run, the average per cent error for a mixture of 14 amino acids in proportions simulating β-lactoglobulin, was –2% in one series and –4% in a second. The maximum variations in individual amino acids ranged from –12 to + 9%. This error, however, can be appreciably reduced by increasing the number of chromatograms.

Cystine is largely or entirely destroyed on two-dimensional chromatograms using S. & S. No. 596 or Whatman No. 4 paper under the conditions described above. It is best determined by a combination of the platinic iodide[9] and Fisher[3] procedures. Proline, hydroxyproline and tryptophan are likewise best estimated by special methods, the first two by the isatin method of Fromageot,[10] the last by a combination of the Voisenet-Rhode p-dimethylaminobenzaldehyde reaction (*cf.*[6]) as applied to paper chromatograms and the area[3] or area-density[4] methods.

Methionine,[†] histidine and tyrosine, which are reasonably accurately estimated by specific chromatographic procedures (*cf.* above) have proven to be valuable for the quantitative estimation of amino acids by the ninhydrin method on two-dimensional chromatograms.

Results. The values given in Table III show the color ratios and approximate quantities of arginine, histidine, lysine, tyrosine, phenylalanine, methionine, serine, threonine, leucines, valine, glycine, alanine, glutamic acid and aspartic acid obtained on less than 0.3 mg of casein N. The figures given in the adjoining column (Table III) are those calculated from casein by conventional chemical and microbiological methods.[6]

Summary. Two methods are described which permit the estimation of colored substances on one-dimensional and two-dimensional paper chromatograms with a minimum expenditure of time and material.

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[*] Experiments which are underway indicate that the concentration of each amino acid on the chromatograms may be estimated within approximately 15% by the maximum color density method, when the color value is read off the appropriate "standard curve" prepared from two dimensional chromatograms which are run *simultaneously* with the unknown solution. As in all colorimetry, it is important to have the color density of the standard as nearly equal as possible to that of the unknown.

[9] Winegard, H. M., Toennies, G., and Block, R. J., *Science*, 1948, **108**, 506.

[10] Fromageot, C., private communication, May 1949.

[†] Cystine and methionine have been determined in casein, lactalbumin and human hair hydrolysates by the area method[3] using platinic iodide[9] as the color reagent and water saturated n-butanol-acetic acid as the developing solvent. The following amounts of cystine and methionine, in grams of amino acid per 16.0 g of nitrogen, were found: casein, 0.3; 3.4, lactalbumin 3.0; 2.6, hair 16.0; 0.6.

Received August 16, 1949. P.S.E.B.M., 1949, **72**.

Prophylactic and Therapeutic Effect of Para-Aminobenzoic Acid and Sodium Salicylate on Experimental Allergic Encephalomyelitis.[*] (17426)

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That encephalomyelitis in experimental animals can be produced by the injection of homologous brain tissue was shown by Rivers, Sprunt, and Berry[1] and confirmed by Ferraro

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[*] Aided by a grant from the National Foundation for Infantile Paralysis.

[†] Helen Hay Whitney Foundation, fellow in rheumatic fever.

[1] Rivers, T. M., Sprunt, D. H., and Berry, G. P., *J. Exp. Med.*, 1933, **58**, 39.

and Jervis.[2] Studies by Schwentker and Rivers[3] and Kopeloff and Kopeloff[4] demonstrated the association of this disease with the production of specific anti-brain antibodies. Recent studies[5-10] using Freund's adjuvants to potentiate and hasten the development of encephalomyelitis against homologous or heterologous brain tissue have provided a working laboratory model of acute disseminated encephalomyelitis. Almost without exception investigators studying this disease of laboratory animals have supported the allergic theory of its pathogenesis.[11] That the sensitizing agent is concentrated in the white matter of the nervous system, but also present in the gray matter seems clear[12,13] Alvord's[14] studies have, further, implicated certain purified phospholipids extracted from brain tissue as at least one of the sensitizing hapten groups capable of producing encephalomyelitis, and Bell *et al.*,[15] have recently shown that calcium acetate precipitation of aqueous suspensions of central nervous tissue removes their encephalogenic properties.

In spite of the apparent relation of experimental encephalomyelitis to a group of human diseases (acute disseminated encephalomyelitis, multiple sclerosis) for which effective therapy is lacking, little work has been reported of attempts to find therapeutic or prophylactic agents capable of interrupting the cycle resulting in allergic central nervous system disease in animals. The long recognized clinical usefulness of salicylates in the treatment of the exudative phase of rheumatic fever has led, however, to a series of investigations demonstrating a pronounced effect of large doses of salicylate on the course of antibody production[16-19] and allergic phenomena resulting from antigen-antibody reactions,[20,21] while Coburn and Kapp[22] showed that salicylate could prevent the *in vitro* precipitation of antigen with specific antibody. Suppression of rheumatic symptomatology with para-aminobenzoic acid has been reported from several clinics and the combination of para-aminobenzoic acid with salicylates has been suggested for the treatment of rheumatic fever. Recent studies indicate that both para-aminobenzoic acid and salicylates are protective against certain allergic or hypersensitive states in rabbits[20] and several rather poorly controlled clinical studies indicate that salicylates might be helpful in certain human encephalitides.[23-25]

In an attempt to prevent the development of the acute allergic encephalomyelitis due to immunization against homologous brain tissue, the following 3 experiments were carried out.

In the first experiment 112 guinea pigs weighing 250 to 325 g each were used. All animals were fed balanced guinea pig rations with supplemental vitamin C. Sodium salicylate medications were given by subcutaneous injection of a sterile 5% solution in distilled water. The para-aminobenzoic acid was administered orally with the drinking water in a concentration of 2.5 mg/cc. Sensitization to homologous brain tissue was carried out by subcutaneous injection of a brain-adjuvant (water in oil) emulsion prepared according to our modification of the method of Freund. Ten grams of sterile fresh guinea pig brain were mixed with 100 cc of normal saline by agitation in a Waring Blendor. Two parts of this brain emulsion were added to two parts of Bayol F (a light paraffin oil) containing 7.5 mg of heat killed *Mycobacterium butericum* per cc and one part Falba (a Pfaltz and Bauer product containing a mixture of hydroscopic lipids from lanolin.) The final mixture was then agitated with a Waring Blendor for 4 periods of 5 minutes each. In this way a stable emulsion was produced, each cc of which contained 2.5 mg of heat killed *Mycobacterium buterium* and 30 mg guinea pig brain. The plan of the first experiment was to begin all medications 8 days prior to the sensitizing injections and to continue the medications for 60 days after the injection of brain and adjuvant. Ten guinea pigs were given para-aminobenzoic acid alone in dosages of .5 g per kilo per day. Twenty were given moderate dosages of salicylate, (.2 g per kilo in one subcutaneous injection each day.) Ten were given larger dosages of salicylate, (.3 g per kilo in divided dosage given 2 times daily), while an additional 20 were treated with a combination of para-aminobenzoic acid (.5 g/K/D) and sodium salicylate (.2 g/K/D). Control groups comprising guinea pigs injected with brain and adjuvants, brain alone, adjuvants alone and adjuvants in addition to salicylate and para-aminobenzoic acid medication were included in the study. Simultaneously, 10 normal untreated guinea pigs fed the same type of rations and given the same routine care as the experimental animals served as additional controls.

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[2] Ferraro, A., and Jervis, G. A., *Arch. Neurol. and Psychiat.*, 1940, **43**, 195.

[3] Schwentker, F. F., and Rivers, T. M., *J. Exp. Med.*, 1934, **60**, 559.

[4] Kopeloff, L. M., and Kopeloff, N., *J. Immunol.*, 1944, **48**, 297.

[5] Morgan, I. M., *J. Bact.*, 1946, **51**, 53.

[6] Kabat, E. A., Wolf, A., and Bezer, A. E., *Science*, 1946, **104**, 362.

[7] Freund, J., Stern, E. R., and Pisani, T. M., *J. Immunol.*, 1947, **57**, 179.

[8] Morrison, L. R., *Arch. Neurol. and Psychiat.*, 1947, **58**, 391.

[9] Jervis, G., and Koprowski, A., *J. Neuropath. and Exp. Neurol.*, 1948, **7**, 309.

[10] Ferraro, A., and Cazzulo, C. L., *J. Neuropath. and Exp. Neurol.*, 1948, **7**, 235.

[11] Stevenson, L. D., and Alvord, E. C., *Am. J. Med.*, 1947, **3**, 614.

[12] Kabat, E. A., Wolf, A., and Bezer, A. E., *J. Exp. Med.*, 1947, **85**, 117.

[13] Morgan, I. M., *J. Exp. Med.*, 1947, **85**, 131.

[14] Alvord, E. C., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 459.

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[16] Swift, H. F., *J. Exp. Med.*, 1922, **36**, 735.

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[19] Homburger, F., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 101.

[20] Sullivan, C. J., Parker, T. W., and Hibbert, R. W., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 508.

[21] Campbell, B., *Science*, 1948, **108**, 478.

[22] Coburn, A. F., and Kapp, E. M., *J. Exp. Med.*, 1943, **77**, 173.

[23] Maciel, Z., *Brazil med.*, 1941, **55**, 804.

[24] Behague, P., *Rev. neurol.*, 1942, **74**, 159.

[25] Ferraz, A. J., *Rev. neurol. e. psiquiret. de São Paulo*, 1944, **10**, 1.

In an attempt to determine the therapeutic effect, if any, of sodium salicylate and para-aminobenzoic acid on experimental disseminated encephalomyelitis the second experiment was carried out. Twelve guinea pigs from among 20 weighing 300 to 500 g, which had been inoculated with the brain-adjuvant emulsion previously described, were selected for treatment after the onset of unequivocal central nervous system disease. Four of these diseased animals were treated with sodium salicylate alone in a dosage of .2 g/K/D, and eight were placed on the combined para-aminobenzoic acid and salicylate regimen described for the animals of experiment 1.

In an attempt to establish the time at which para-aminobenzoic acid and salicylate became effective in the prophylaxis of acute disseminated encephalomyelitis, the third experiment was carried out. Forty guinea pigs weighing 425 to 600 g were studied. Ten of these animals injected with the brain adjuvant emulsion and given no further treatment served as controls, while one of the remaining guinea pigs was started on treatment with the para-aminobenzoic acid and salicylate combination previously mentioned on each successive day beginning eight days prior to the injection of all the animals with the brain adjuvant emulsion and continuing for 22 days thereafter.

Results. The results of Experiment 1 are summarized in Fig. 1. Approximately 90% of 22 guinea pigs receiving injections of guinea pig brain and adjuvants developed symptoms of central nervous system disease between 13 and 27 days after the injection. Most frequently the disease began with spasticity of the hind legs followed within a few days by flaccid paralysis and encephalitis. Tremors, nystagmus, and convulsions were prominent symptoms in the late stages of the disease. Seventeen of these control animals died from encephalomyelitis between 19 and 42 days after the initial injection of the water in oil emulsion of guinea pig brain. The duration and course of the disease after onset were variable with exacerbations and remissions being noted occasionally. Once unequivocal evidence of central nervous system involvement became apparent, the animals usually died. In several guinea pigs, however, the disease became stationary for long periods. A few animals recovered, some with sequellae,

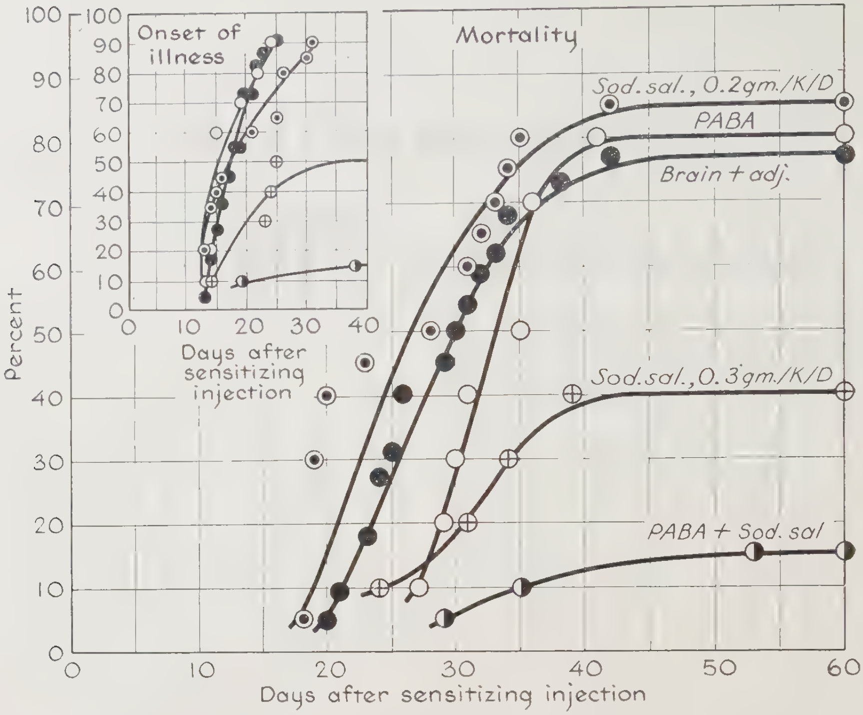

FIG. 1.

Table of mortality curves of 112 guinea pigs of experiments I and II. Insert shows onset of illness in same series. See text.

others with none. Cultures of the central nervous tissue removed with careful aseptic precautions from moribund, paralysed, encephalitic animals showed no growth on blood agar plates. Neither were attempts to demonstrate a virus in the involved nervous tissue by intracranial and intraperitoneal mouse passage, intracranial and intraperitoneal guinea pig passage, nor corneal and intraocular rabbit passage successful.

While premedication and medication with moderate doses of either sodium salicylate or para-aminobenzoic alone failed to alter the incidence or the course of the central nervous system disease, larger total dosages of sodium salicylate seemed to delay the time of onset and reduce both the incidence and mortality rate. Even more striking were the results obtained when a combination of para-aminobenzoic acid and sodium salicylate was used for the prophylaxis and treatment of this experimental disease. Fifteen per cent of the guinea pigs treated in this way developed symptoms and signs of encephalomyelitis after injection with brain and adjuvant and only 10% died. Injection of either adjuvants or homologous brain alone was well tolerated by the controls. During the period of the experimental study, 10 normal guinea pigs remained in good health showing substantial growth on the same dietary regimen provided for the experimental animals.

The therapeutic effectiveness of sodium salicylate and para-aminobenzoic acid was tested on a group of 12 guinea pigs selected on the first day of central nervous system involvement following 14-30 days after the injection of brain and adjuvants. No alteration in the course of the disease nor apparent amelioration of symptoms was produced in any of the animals treated with sodium salicylate or combined sodium salicylate and para-aminobenzoic acid. One guinea pig in the group treated with para-aminobenzoic acid and salicylate showed several remissions followed by exacerbations and ultimately survived. The remaining seven guinea pigs of this group and all 4 treated with sodium

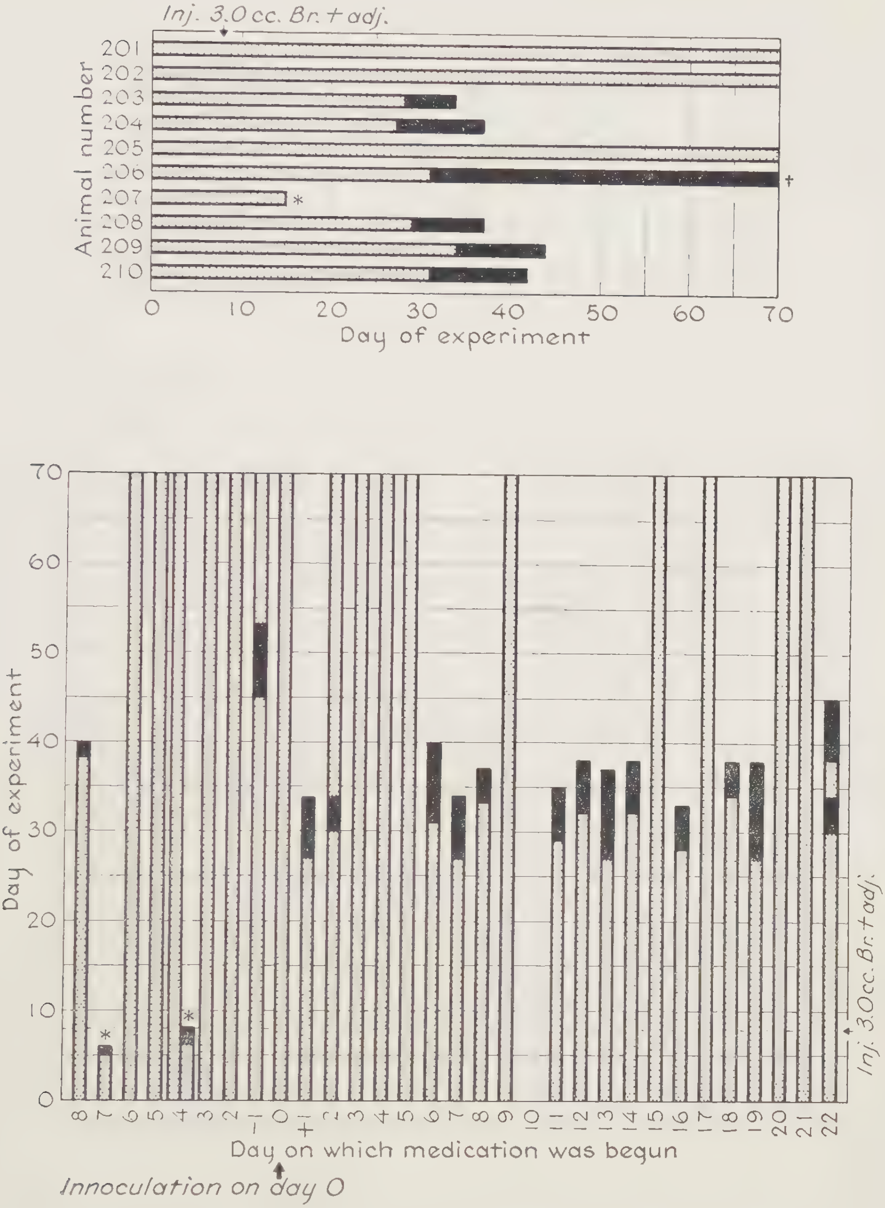

FIG. 2.

Chart showing individual clinical histories of 10 control animals and 30 which commenced medication at various days, before and after the sensitizing injection. Stippled columns indicate disease-free period, solid columns represent period of illness. Termination of the columns indicates death of animal. Asterisks indicate death of animals from causes other than the experimental disease. Animal 206, long sick, was killed at the termination of the experiment.

salicylate alone died between 4 and 18 days after the initial symptoms and beginning of treatment. This result was not significantly different from that occurring among the control guinea pigs.

The results obtained in Experiment 3 are indicated in Fig. 2. Here again evidence of a protecting effect of combined para-aminobenzoic acid and salicylate prophylaxis is apparent. The control group (Fig. 2) which received brain plus adjuvant but no medication, showed 5 out of 9 dying (55%) with an incidence of 6 out of 9 acquiring the disease (67%). In the treated series, little morbidity was seen in the group in which medication was commenced before the fifth day following injection. Past that point, however, the incidence of symptoms and death appear not significantly different from the control. These results suggest that protection against experimental allergic encephalomyelitis is effective when sodium salicylate and para-aminobenzoic acid medication are begun early. It would seem from these data that if medication is delayed for several days following the injection of the brain and adjuvant no protecting effect can be demonstrated. In this series significant protection was apparent among the guinea pigs whose treatment was initiated prior to the fifth day following injection with brain and adjuvant, while those whose treatment was begun later were not protected.

Although the pathologic studies of the series of animals will be the subject of another report, a brief mention of the results obtained are of interest here. The central nervous system changes include scattered foci of inflammatory reaction with subependymal and perivascular concentrations being characteristic. The marked plasmacellular component of the inflammatory reaction previously noted by others[8-10] was also apparent in our material and is in keeping with an hypothesis previously expressed[26-28] that local plasma cell accumulation in the central nervous tissue, as well as elsewhere, indicates the operation of antigen-antibody reactions.

Discussion. Many problems are posed by these results. Although in these experiments serum antibodies were not studied, we assume that the disease with which we are dealing is an expression of the specific antibrain hypersensitivity. A number of investigators have demonstrated the association of the nervous disease produced in this way with the development of antibrain antibodies.[3,4,32] Because of our previous experience[29,30] with the activation of latent virus in the central nervous system by antigen-antibody reaction and because the symptoms of nervous system involvement in this disease simulate in certain respects known virus disease of guinea pigs, mechanisms involving virus invasion were suspected of operating in this situation. It seems reasonably certain, however, that our futile search for virus in the tissues of these animals as well as negative searches for virus in the central nervous tissues of similar animals by others render this possibility very unlikely. The constant two to four week latent period following injection of antigen is compatible with the allergic hypothesis of the pathogenesis of this encephalomyelitis.

The protection afforded by the combination of salicylates and para-aminobenzoic acid can probably be explained through operation of known actions of these drugs. An *in vivo* as well as *in vitro* effect of salicylates in blocking antigen-antibody reactions seems well established. In addition, the weight of the evidence would indicate that large doses of salicylates act in some way to reduce antibody formation. That moderate dosages of salicylates do not affect antibody response to the streptococcus and its products, was pointed out by Rantz, Boisvert, and Spink[31] while the effect of larger dosages of salicylate on the antibody response to a variety of antigens has been demonstrated. This relationship might help to explain the lack of protection against acute allergic encephalitis noted when sodium salicylate in a dosage of .2 g/K/D was used while protection was obtained with larger dosage of salicylate as well as when the smaller salicylate dosage was combined with para-aminobenzoic acid.

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[29] Good, R. A., and Campbell, B., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 305.

[30] Good, R. A., and Campbell, B., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 82.

[31] Rantz, L. A., Boisvert, P. J., and Spink, W. W., *Science*, 1946, **103**, 352.

[32] Kuprowski, H., and Jervis, G. A., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 472.

Para-aminobenzoic acid may by itself have anti-anaphylactic properties[20] and the effect noted here might be an expression of a synergistic antianaphylactic action. It is entirely possible, however, that the decreased elimination of sodium salicylate in the urine in the presence of para-aminobenzoic acid[33] results in higher, more effective, blood levels of salicylate than when this drug is given alone. If so, other acid salts, like ammonium chloride, might also result in a comparable enhancement of the salicylate effect. While toxic reactions to salicylates and para-aminobenzoic acid may preclude the safe attainment of effective levels in the treatment of human disease, further studies are indicated to elucidate the underlying mechanisms of this protecting effect. There is reason from this and other studies to believe that a less toxic drug with antianaphylactic properties in common with salicylates might be an important therapeutic and prophylactic agent in human disease.

The now extensive studies of auto-immunization phenomena in diseases of experimental animals demand further attempts to establish the operation of such mechanisms in the pathogenesis of human disease in spite of the technical difficulties involved. It seems obvious that this phenomenon could be at work in post infectious states, the pathogenic mechanisms of which remain obscure. Further studies on rheumatic fever, post infectious leukoencephalitis, multiple sclerosis, sympathetic ophthalmia, and glomerulonephritis might, indeed, reveal that autosensitization is a common denominator for many otherwise unrelated diseases.

Summary and conclusions. 1. Progressive acute disseminated encephalomyelitis was produced in 90% of a group of guinea pigs by the subcutaneous injection of water in oil emulsions of homologous brain tissue plus adjuvants.

2. While prophylaxis and treatment with moderate dosages of either para-aminobenzoic acid or sodium salicylate alone provided no protection of guinea pigs against experimental allergic encephalomyelitis, the use of larger dosages of salicylate as well as combined salicylate and para-aminobenzoic acid therapy were effective in preventing this disease.

3. Effective prophylaxis was demonstrated only when the medication was started before or shortly after the sensitizing injection of brain tissue was given.

4. No therapeutic effect of sodium salicylate alone or combination of para-aminobenzoic acid with sodium salicylate could be shown.

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Received August 18, 1949. P.S.E.B.M., 1949, **72**.

The Initial Uptake of C^{14}-Labelled Methadone by Rat Tissues.* (17427)

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Recently it has been shown in this laboratory that within one hour after a subcutaneous injection of 10 mg/kg of C^{-14} labelled methadone (methadone*) into rats, the concentration of the drug in the brain is relatively low.[1] A fairly rapid mobilization of the drug from the site of injection is indicated by the work of Eisenbrandt, *et al.*[2] who found that within 10 minutes after such an injection,

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* Supported, in part, by a grant from the National Institutes of Health, Bethesda, Md.

† On sabbatical leave from the University of Kansas City, Kansas City, Mo.

[1] Elliott, H. W., Chang, F. N-H., Abdou, I. A., and Anderson, H. H., *J. Pharm. and Exp. Therap.*, 1949, **95**, 494.

[2] Eisenbrandt, L. L., Abdou, I. A., and Adler, T. K., *Fed. Proc.*, 1949, **8**, 288.

radioactivity appeared in the bile. In view of this rapid mobilization it was desirable either to establish or to rule out the possibility that an initial high concentration of methadone in the brain is responsible for the rather profound effects on the central nervous system.

The study was extended to include determination of the rate of accumulation of methadone* by the adrenals and the liver, both of which were found by Elliott, *et al.*,[1] to contain high concentrations after one hour, and to correlate these findings with the rate of accumulation in the blood. In addition, portions of the sciatic nerve were analyzed to check the possibility that analgesia might be produced by concentration of methadone* in the peripheral nervous system.

Methods. The C^{-14} labelled methadone HBr used in this study has been described previously.[1,3] Eight young adult rats (4 male and 4 female) of the Slonaker-Wistar strain weighing between 175-250 g and maintained on a diet of Purina Dog Chow and tap water were used in this study. Each was given 10 mg/kg of methadone* by subcutaneous injection into the left hind leg. After 10, 20, 30, or 40 minutes one male and one female were sacrificed. Ether was administered immediately before sacrifice, and a blood sample was obtained from the abdominal aorta.

Cerebrum, adrenals, liver and portions of the sciatic nerve from the uninjected (right) leg were dried to constant weight at 60°C. Tissue water content was calculated from the wet-dry weight difference.

Preparation of tissues for combustion has been described previously[1]. The method of combustion was that of Van Slyke and Folch[4] as modified by Skipper, *et al.*[5] The combustion apparatus used has been described by Entenman, *et al.*[6] Preparation of radioactive samples of $BaCO_3$ for counting was similar to that used previously in this laboratory.[1]

Although there is evidence that methadone can be metabolized by the body,[7,8] the possible metabolic products have not been identified. However, the labelled carbon atom incorporated in the methadone* used in these studies was not oxidized to CO_2.[1] Therefore, for the sake of simplicity the data are reported as micrograms of methadone*.

Results. A comparison of the concentration of radioactivity in whole blood with that in the plasma indicated a dilution of the former by the red blood cells. To establish this point definitely, the red blood cells were centrifuged, washed twice with saline and analyzed. No radioactivity could be found, thus indicating that within the period studied (between 10 and 40 minutes) the methadone* in the blood was confined to the plasma.

Furthermore, it seemed desirable to establish whether or not accumulation of the drug in the tissues was merely a reflection of accumulation in the plasma water. For this reason calculations were made on the basis of concentration per gram of tissue water in addition to the usual method of reporting such data.

The concentration of methadone* in the tissues studied is summarized in Table I. Values for the sciatic nerve are omitted from the table since at no time did the nerve contain significant amounts of radioactivity.

Discussion. The rapid appearance of radioactivity in the plasma indicates the importance of the plasma as a transport mechanism in mobilizing a subcutaneous injection of methadone.* It is of interest to note that the concentration in the cerebrum was of the same order of magnitude as that of the plasma, and also that the drug did not accumulate in the sciatic nerve. The low amounts in the cerebrum dispute the thesis that analgesia is

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[3] Tolbert, B. M., Christenson, F., Chang, F. N-H., and Sah, P. P. T., *J. Am. Chem. Soc.*, in press.

[4] Van Slyke, D. D., and Folch, J., *J. Biol. Chem.*, 1940, **136**, 509.

[5] Skipper, H. E., Bryan, C. E., White, L., Jr., and Hutchinson, O. S., *J. Biol. Chem.*, 1948, **173**, 371.

[6] Entenman, C., Lerner, S. K., Chaikoff, I. L., and Dauben, W. G., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 364.

[7] Richards, J. C., and Boxer, G. E., paper presented to the Division of Medicinal Chemistry of the Am. Chem. Soc. meeting of Sept. 18-23, 1949.

[8] Way, E. L., Signorotti, B. T., and March, C. H., to be published.

TABLE I.
Tissue Concentration of Radioactivity in Terms of Micrograms of Methadone* per Gram.

Rat No.	Time after inj., min.	Cerebrum		Plasma		Adrenals		Liver	
		per g water	per g dry tissue	per g water	per g dry tissue	per g water	per g dry tissue	per g water	per g dry tissue
16	10	0	0	0.4	4.9	0	0	2.4	5.4
21	10	0	0	0.05	5.5	11.2	16.9	4.1	9.5
17	20	2.3	8.3	0.9	10.5	16.0	42.8	18.8	49.2
22	20	6.0	20.0	2.6	27.9	39.7	68.4	10.9	25.7
18	30	0	0	0.9	9.8	22.9	26.4	20.1	47.5
23	30	3.3	11.9	1.2	14.5	27.4	51.5	13.4	33.6
19	40	3.6	13.0	2.0	24.9	48.5	93.0	25.5	60.7
24	40	2.5	8.6	2.5	30.0	45.0	96.5	30.8	79.4

induced by an initial high concentration of the drug in the brain.

In contrast to the cerebrum, both the liver and adrenals showed a marked increase in concentration with time; the rate of increase in the adrenals was more pronounced. These differences, obtained even when calculated on the basis of tissue water, suggest a definite mechanism for accumulation in these tissues.

Summary. Based on the determination of radioactivity for periods of 10, 20, 30, and 40 minutes following a subcutaneous injection of 10 mg/kg of C^{14} labelled methadone HBr into rats, it was found that:

(a) Methadone* makes its appearance in the blood within 10 minutes, and is confined to the plasma.

(b) There was no activity in the cerebrum before 10 minutes, and even thereafter the concentration is very low. The rate of accumulation roughly parallels that of the plasma indicating no special affinity of the brain for the drug.

(c) The adrenals and liver show a marked ability to accumulate methadone* at a rate in excess of that shown by either the cerebrum or plasma.

(d) There was no activity in the sciatic nerve within the period studied.

Received August 22, 1949. P.S.E.B.M., 1949, **72**.

A Nervous Syndrome Produced with Phenylalanine and Methionine.*

(17428)

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During studies on the metabolic interrelationships between threonine, phenylalanine, tryptophan and niacin, it was noted that animals fed a niacin-tryptophan deficient ration supplemented with 0.208% DL-phenylalanine and 0.2% DL-methionine for a period of 12 weeks developed an interesting nervous syndrome. The symptoms include an arched back with extended legs in a rigid condition. The head oscillates vertically almost continuously, and the rate of movement increases until the animal apparently becomes fatigued. After a short rest period, this movement reoccurs. The animal may show a slow rotary motion of the body and periodically it may move backwards at a rapid rate. Death results 36 to 50 hours after the onset of the symptoms. Blood pressure measurements on one animal with very severe symptoms gave normal values. Autopsy of all animals failed to reveal any gross abnormalities in the structure of any of the organs; however, the stomachs of some were very slightly bloated.

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* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by grants from the National Dairy Council, on behalf of the American Dairy Association, and the Nutrition Foundation, Inc.

The animals fed niacin or tryptophan in the ratio in addition to the added DL-phenylalanine and DL-methionine were normal. The rations supplemented with 0.2% L-cystine plus 0.208% DL-phenylalanine instead of 0.2% DL-methionine plus 0.208% phenylalanine did not produce this syndrome. Even though DL-threonine is generally much more active as a growth inhibitor in the 9% casein ration, it did not produce these symptoms, when either L-cystine or DL-methionine was added to the ration. Apparently there is a definite relationship between methionine and phenylalanine which is responsible for the nervous syndrome.

An experiment was designed (Table I) to determine what levels of methionine and phenylalanine would be required to shorten the period for onset of the syndrome, and what compounds could be used to cure it. The composition of the basal ration was the same as that used in previous studies.[1] The combinations of 0.208% DL-phenylalanine plus 0.2% DL-methionine, 0.208% DL-phenylalanine plus 0.4% DL-methionine and 0.416% DL-phenylalanine plus 0.4% DL-methionine all appeared to produce the nervous syndrome in the same period of time. The administration of niacin or tryptophan by stomach tube, the injection of niacin, or the feeding of tryptophan solution orally by dropper, after the animals exhibited severe nervous symptoms, did not produce a cure. On the other hand if the level of casein in the ration was doubled at the onset of the early symptoms of the syndrome, the animals quickly recovered. It is important that niacin or tryptophan protected the animals when given from the beginning, but they were inactive when given after the syndrome had reached a definite stage.

TABLE I. Response of Individual Rats to Various Supplements Administered After Onset of Nervous Syndrome.

Supplement	Time required for symptoms to appear, wk	Survival time after appearance of symptoms, hr	Treatment
0.208% DL-Phenylalanine + 0.2% DL-methionine	12	56	No treatment.
” ”	12.5	50	100 mg DL-tryptophan in 25% alcohol sol. fed (ml containing 20 mg) by stomach tube 48 hr. 1 ml fed every 8 hr.
” ”	7	24	No treatment.
” ”	9	60	12.5 mg niacin in 25% alcohol sol. fed (ml containing 12.5 mg) by stomach tube. After 24 hr period, 8 mg niacin (8 mg/ml) in aqueous sol. inj.
” ”	11	—	18% casein ration fed; complete recovery followed.
” + 0.4% ”	3	24	No treatment.
” ”	8	36	No treatment. Blood pressure normal (125 mm). Brain removed for pathological studies.
” ”	11	—	18% casein ration fed; complete recovery followed.
0.416% ” ”	9	36	25 mg DL-tryptophan in 25% alcohol fed by dropper 48 hr. At autopsy all tissues appeared normal.
” ”	11	—	18% casein ration fed; complete recovery followed.
” ”	—	—	The rat removed at end of 13 wk.

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[1] Hankes, L. V., Henderson, L. M., Brickson, W. L., and Elvehjem, C. A., *J. Biol. Chem.*, 1948, **174**, 873.

The apparent inability of the animals to control their movements indicated that the cells in certain areas of the brain may have been affected. The levels of methionine and phenylalanine in the rations may have altered the ratio of amino acids in the brain cells[2] to such an extent that permanent changes in the enzyme or metabolic systems of the cells took place. Furthermore, the observation that the symptoms were not reversible by feeding or injecting niacin would substantiate this conclusion, since this compound prevented the onset of the nervous symptoms, when it was present in the ration. A gross examination of the brains of several animals did not reveal any abnormalities.

It is believed that this is the first time a syndrome of this nature has been observed in experiments with rations containing an imbalance of amino acids. There are known human diseases such as cystinuria,[3,4] alkaptonuria,[5] tyrosinosis,[6] and phenylpyruvic oligophrenia,[7,8] which have been related to inborn errors of amino acid metabolism. In these diseases normal metabolic processes involving phenylalanine and tyrosine are either partially or completely inhibited, and the disorders are generally recognized by the persistent excretion of abnormal urinary constituents.[9,10]

When our results are considered in the light of faulty amino acid metabolism, it is feasible that the effects observed were due partially to a limiting ability of the animal to metabolize phenylalanine. It is also possible that the causative agent in this syndrome was the D isomer of this amino acid, since it has been observed that the growth of rats was reduced drastically when this isomer was supplemented at low levels in a 9% casein ration.[1]

Summary. Animals fed a niacin-tryptophan deficient ration supplemented with 0.208% DL-phenylalanine and 0.2% DL-methionine for a period of 12 weeks developed a nervous syndrome. Generous quantities of tryptophan or niacin in the ration protected the animals from this syndrome, but neither compound cured the symptoms once they had progressed beyond a given stage.

We are indebted to Merck and Co., Inc., Rahway, N. J., for the supply of crystalline B vitamins, and to the Abbott Laboratories, North Chicago, Ill., for the generous supply of haliver oil.

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Received August 25, 1949. P.S.E.B.M., 1949, **72**.

A Modified Diphenylamine Procedure for Fructose or Inulin Determination. (17429)

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The use of inulin clearance as a measure of glomerular filtration rate has prompted the appearance of a number of methods for the determination of inulin or fructose, the majority of which are based either on the Seliwanoff reaction[1] or the reaction with

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* This work was aided by a grant from the Commonwealth Fund.

diphenylamine described by Ihl.[2] The hexoses when heated in the presence of strong acids form hydroxymethylfurfural and this in turn reacts to form a colored condensation product with either resorcinol or diphenylamine. Although the ketohexoses react much more readily than the aldohexoses, glucose in the concentrations normally found in plasma will yield a significant amount of color. If the plasma glucose remains constant this error is eliminated by the use of a control blank. Since this is not generally the case, the usual procedure has been either to remove the glucose by fermentation or to determine it in each sample and make a correction.

The amount of color produced by fructose or inulin as compared to that produced by glucose varies somewhat with different methods. Factors which influence this ratio are, (1.) acidity of the reacting solution, (2.) concentration of resorcinol or diphenylamine, (3.) duration of heating and (4.) temperature at which the reaction is carried out. While a decrease in any of these factors will decrease the speed of the reaction, the glucose color production is diminished to a greater extent than the fructose. Corcoran and Page,[3] using a diphenylamine procedure at 100°C, found a fructose to glucose color ratio of 40 and say, "Error from this source is abolished by the compensating blank when blood glucose is constant, and the error amounts to only 0.5 mg of levulose per 100 cc with glucose concentrations varying 20 mg per 100 cc." Using their method we have found the ratio to be between 20 and 25; Corcoran has recently informed us that they now find this lower ratio. Findley and White[4] found a ratio of 25; since then it has been found to be more nearly 20. Harrison,[5] who has improved the technic by substituting glacial acetic acid for ethyl alcohol as the solvent for diphenylamine, does not state his ratio, but says, "It is necessary to remove glucose from the blood plasma." Using his method we have found the ratio to be 8. Roe, Epstein and Goldstein,[6] using a resorcinol method, have recently reported a fructose to glucose color ratio of about 2000 at various concentrations of glucose; such a ratio would render any errors due to glucose fluctuations insignificant. We have not been able to confirm their finding. Using their method and working with aqueous glucose solutions containing 0.0625 mg/ml to to 0.5 mg/ml and with fructose solutions containing 0.005 mg/ml to 0.03 mg/ml we have found the color ratio to be approximately 50. Color productions by glucose and by fructose are additive when the two sugars are determined together. We have found also that the color produced by this reaction is quite sensitive to light, either artificial or daylight, and that there can be a difference of as much as 15% between duplicates even when the samples are read within the 30 minutes recommended in their paper, unless special precautions are taken to maintain light exposure at a minimum and uniform for all samples.

In an effort to find a set of conditions which would yield a high fructose to glucose color ratio we have tried many combinations of diphenylamine and HCl with different heating times and temperatures. The following procedure has produced the highest fructose to glucose ratio and yielded the best agreement between duplicates. Although we have not been able to verify Roe, Epstein and Goldstein's exceedingly high color ratio, it still appears to be a satisfactory method since even a ratio of 50 is higher than with previously reported methods; in our hands it has yielded accurate results when colored samples are protected from light. It is still felt, however, that the method presented here has some advantages in that the color ratio is somewhat higher and the color produced is stable. It further avoids the unpleasantness involved in handling their highly concentrated HCl reagent.

Method. Reagent. Dissolve 3 g of diphenylamine† in 100 ml of glacial acetic acid and add

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TABLE I.
Optical Densities × 10^3 Produced by Fructose, Inulin, and Glucose Solution.

	Fructose, μg/2 ml					Glucose, mg/2 ml	Fructose / glucose
	10	20	30	40	60	1	
1		145 ± .5(6)	222 ± .6(6)	289 ± 1.0(6)	433 ± 1.0(6)		
2		142 ± .5(5)	220 ± 1.0(6)	285 ± .9(6)	436 ± .8(6)	117 ± .7(4)	62
3	89 ± .5(5)	147 ± .5(5)	221 ± .7(5)	291 ± .8(5)	447 ± .5(5)	114 ± .6(4)	65
4	82 ± .3(6)	154 ± .5(5)	236 ± .2(5)	309 ± .9(4)	460 ± .4(4)	124 ± .5(3)	63
5	77 ± .5(3)	148 ± .7(3)	220 ± .7(3)	294 ± 1.0(3)	448 ± .5(3)	118 ± .0(2)	63
6	74 ± .5(3)	152 ± .4(3)	228 ± 2.0(3)	295 ± 1.0(3)	445 ± 1.0(3)	114 ± 1.0(3)	66
7	72 ± .0(3)	148 ± .7(3)	230 ± 2.0(3)	305 ± 1.0(3)	457 ± 2.0(3)	120 ± 1.0(3)	63
			Inulin μg per 2 ml.				
8			229 ± .7(10)				
9		142 ± .7(6)	204 ± .4 (6)	284 ± .7(4)	426 ± 1.0(6)		

The data of a horizontal line were obtained with a single heating. The figure following the ± is S.E. for the samples of a given concentration in the same heating; the figures in parentheses give n, the number of samples. Ten 30 μg fructose samples run simultaneously with the 10 inulin samples of line 8 gave an average optical density × 10^3 of 234 ± 0.8, indicating complete hydrolysis of the inulin. The inulin densities in line 9 are somewhat lower than those usually obtained because some diphenylamine had precipitated after the reagent had stood 2 days in the refrigerator; simultaneous fructose determinations (not shown in table) were also correspondingly low.

60 ml of conc. HCl. This is the same reagent used by Harrison. Prepare fresh each day; if kept in a refrigerator some of the diphenylamine will precipitate and it is difficult to redissolve it. Use of this reagent after partial precipitation of diphenylamine yields a calibration curve which is lower than one obtained with fresh reagent.

Procedure. Pipette 2 ml of a 1:10 plasma filtrate[7] or appropriate urine dilution into a test tube 15 x 125 mm. Add 4 ml of diphenylamine reagent and *mix well* with swirling. Since the reagent has a high viscosity, care must be taken that pipette drainage is adequate. For plasma determinations use as a blank 2 ml of a 1:10 filtrate of plasma obtained just prior to the injection of inulin; 2 ml of water is used for urine blanks. Place in a rack and heat in a water bath at 75°C for 60 minutes. The bath used had a thermoregulator sensitive to ± 0.7°C at 75°C; a mechanical stirrer insured uniform heating of all samples. At the end of the heating period place rack in an ice bath for 1 to 2 minutes. If the tubes are kept too long at the lower temperature there may be some precipitation of diphenylamine. Read the samples in a photoelectric colorimeter with a 640 filter or a spectrophotometer at 640 mμ.

When 2 ml samples containing 10 to 60 μg of fructose or inulin in water solution are used, optical density plotted against concentration yields a straight line. This corresponds to a range from 5 to 30 mg per 100 ml of plasma since 2 ml of a 1:10 filtrate are used. Separate calibration curves may not superimpose. Since the curves are straight lines, the inclusion of a standard with each set of determinations makes it possible to correct the values read from the calibration curve.

Table I shows the optical densities obtained with various concentrations of fructose, inulin and glucose. There is good agreement between samples of the same concentration in any one heating batch (represented on a horizontal line). Within the range of concentrations shown in Table I a given amount of fructose yields 64 times as much color as the same amount of glucose; this same ratio holds when fructose color development is compared with that of glucose in concentrations of 0.125 and 0.250 mg/ml (not shown in table). In our experience the plasma glucose level of fasted subjects does not depart more than 10 mg % from the blank value during

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† Since this paper went to press we have been using 2.5 g of diphenylamine and have been keeping the tubes covered while they are in the water bath. This has prevented crystallization of diphenylamine.

[7] Somogyi, M., *J. Biol. Chem.*, 1930, **86**, 655.

a clearance experiment; the fluctuation is usually less than this. With a plasma inulin level of 15 mg % a 10 mg % departure from the blank would produce an error of one % in the inulin value determined.

A series of determinations on different solutions containing both glucose and fructose were run in the same heating batch with the corresponding separate glucose and fructose concentrations. The ratios of the observed additive optical densities to the calculated were 0.993, 0.993, 1.003, 1.007, 1.015, 0.994, 0.994, 0.985, 1.010, 1.006, 1.033, 1.006, 1.020 and 0.987.

Summary. A modification of Harrison's diphenylamine method for fructose or inulin determination is described, heating being carried out at 75°C for 60 minutes. With this procedure the ratio of fructose to glucose color production is 64, as compared with a ratio of 8 at 100°C for 30 minutes. Inulin hydrolysis is complete, the standard error of the method is less than 1 percent, the color is light stable and the low color production by glucose permits one to omit fermentation except in the presence of unusual glucose fluctuations.

Received August 28, 1949. P.S.E.B.M., 1949, **72**.

False Positive Trichina Precipitin Reactions in Neoplastic Disease. (17430)

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(Introduced by C. P. Rhoads)

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Because of high fever, palpebral edema, painful inflammation of feet and hands, and eosinophilia of 5% in a child with atypical lymphosarcoma, a trichina precipitin test was performed. The reaction was strongly positive in a dilution of 1:1280, the highest titre routinely tested. The clinical signs rapidly subsided during treatment with 4-aminopteroylaspartic acid, a trichina complement-fixation test was negative, and x-ray examination demonstrated lesions of bone of the "moth-eaten" type commonly seen in acute leukemia. Hence, although the precipitin test remained unchanged, there was no longer any clinical reason to consider a diagnosis of trichiniasis and it was felt that the trichina precipitin reaction was a false positive.

This occurrence of an apparently false positive trichina precipitin test in a patient with lymphosarcoma led to a study of sera from other patients with lymphosarcoma, Hodgkin's disease, leukemia, and other neoplastic diseases. Positive reactions were so frequently seen that it seemed desirable to report the phenomenon.

Method. Sera were obtained from hospitalized and clinic patients and personnel of Memorial Hospital. Three categories were studied: (1) those neoplastic diseases which involve principally the reticuloendothelial system (Hodgkin's disease, lymphosarcoma, the leukemias, and multiple myeloma), (2) widespread metastatic disease of other tissue, both carcinomas and sarcomas, (3) apparently healthy controls. Group 2 was included as controls who showed advanced and debilitating disease comparable to that seen in the first group.

Precipitin tests were performed by the standard technic of the Bureau of Laboratories of the New York City Department of Health. The antigen consisted of a saline extract of *Trichinella spiralis* larvae obtained from infected rat muscle by digestion with pepsin and hydrochloric acid. Undiluted serum was pipetted into small bore test tubes and overlaid with antigen in dilutions of 1:160, 1:320, 1:640, and 1:1280 on a basis of weight of dried larvae. A positive test was indicated by the formation, after two hours at room temperature, of an opaque ring. A

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* Fellow of the American Cancer Society, recommended by the Committee on Growth of the National Research Council.

TABLE I.
Results of 171 Trichina Precipitin Tests from 128 Persons With and Without Neoplastic Disease. Numerals indicate number of patients, not number of serum samples. Figures in parentheses indicate patient on whom skin tests were done (all negative), and those in brackets indicate patients on whom complement fixation tests were done (all negative).

Diagnosis	Total No. patients	Trichina precipitin titre					
		Neg.	160	320	640	1280	variable
Hodgkin's disease	13	1			4	8[1](1)	
Lymphosarcoma	14	5		2	2	4[2]	1
Acute leukemia	25	10			2	7(3)	6
Chronic leukemia	13	10		1	1	1	
Multiple myeloma	3	2			1		
Nieman-Pick's disease	1	1					
Osteogenic sarcoma	3				1(1)	1	1
Wilm's tumor	1				1		
Rhabdomyosarcoma	1	1					
Bronchogenic carcinoma	3				1	2	
Cancer of breast	8	4	2	1	1		
Cancer of prostate	5	3		2			
″ ″ tongue	1	1					
″ ″ uterus	2				1	1	
Normal healthy adults	26	26					
Normal healthy children	9	9					

titre of 320 is generally considered significant for diagnosis of trichiniasis.

Results. Tests have been done on 171 serum samples from 128 persons. Results are summarized in Table I.

All normal controls tested[†] (26 adults and 9 children) had negative reactions.

Of 13 patients with Hodgkin's disease, 12, in all stages of disease, had highly positive reactions. The negative reactor was a 10 year old boy; all others were adults. Of 14 patients with lymphosarcoma, 5 had negative reactions. These 5 were all adults in good condition. Seven (including 4 children) had positive reactions in a high titre and all of these patients had terminal and rapidly progressive disease. One case of lymphosarcoma in good condition and one in the terminal stage of the disease gave intermediate titres (320). Of 13 patients with chronic leukemia (7 myeloid, 5 lymphatic), only 3 had positive titres. Each of these was in the terminal phase of myeloid leukemia and showed a large percentage of blasts in marrow and peripheral blood. Results in 25 cases of acute leukemia were variable. Nine patients with acute leukemia had negative titres on all serum samples tested, 8 had consistently positive titres, and 8 had variable titres on serial specimens. Those who consistently had high titres all died without remission. Most patients who had hematologic remissions (on treatment with antagonists of pteroylglutamic acid) had either consistently negative titres (5 patients), or on serial tests reverted from positive titres to negative (3 patients).

No generalization can be made concerning patients with other types of neoplastic disease. Of the 3 patients with bronchogenic carcinoma and 3 with osteogenic sarcoma who were tested, all gave high positive titres. Thirteen patients were tested who had metastatic carcinoma of breast or of prostate. Only one of these had a high titre and 7 were negative. It is noteworthy (in view of the positive titres found in bronchogenic and osteogenic neoplasms) that most of the patients with these 2 diagnoses had metastases to bones or lungs or both. Other diagnoses are represented by too few cases to merit discussion.

Three sera showing highly positive precipitin titres (2 patients with lymphosarcoma and one with Hodgkin's disease) were tested by John Bozicevich of the National Institutes of Health for complement-fixing antibodies with trichina antigen. All 3 were negative. Skin tests were performed with trichina antigen diluted 1:8000 on 5 patients having strongly positive precipitin reactions. All were negative (Table I).

[†] Twelve of the adult control sera were obtained by Dr. Joseph J. Bunim of The New York University Medical School.

Discussion. The frequency of false-positive reactions in the presented data naturally raises questions as to the significance of these results, and the cause and mechanism of the reaction.

Reliability of data. All tests were performed by one person (A.E.T.) and were done without any knowledge of the diagnosis. The reproducibility of results in serial specimens from many of the patients (chiefly those with Hodgkin's disease, lymphosarcoma, and leukemias) indicate that the data as presented are reliable. Thirty-six patients had at least duplicate tests and only 10 have showed variation of more than one dilution. Those who showed variation in titre also showed concurrent variations in the disease process.

Reliability of the method. In established trichiniasis the precipitin test shows a fairly high degree of reliability. In recent acute trichiniasis most workers have reported over 90% positive reactions (reviews of literature by Gould,[1] and by Shookhoff *et al.*[2]).

False positive reactions are known to occur. Gould[1] reports 51 patients in whom trichina could *not* be demonstrated at autopsy and in whom precipitin tests had been performed. Ten of these (19.8%) had a positive reaction at a dilution of 1:100. The diagnoses of these patients are not reported. Bachman *et al.*[3] studied 857 sera from an area where trichiniasis is not endemic and found 8.4% apparently-false positive tests in patients with a wide variety of non-neoplastic disease. Augustine and Theiler[4] in a similar study found 18 apparently false-positive reactions (12%) among 115 patients and felt that the false positives were related to medications (quinine, mercury, bismuth, and arsenic). There was no apparent relation to these same medications in Bachman's series. A single case of periarteritis nodosa reported by Reimann, Price, and Herbut[5] showed a false positive trichina precipitin test. Bassen, Thomson, and Silver[6] reported false positive precipitin tests in 5 cases of infectious mononucleosis.

Significance of present data. It is not probable that the reactions reported here indicate subclinical trichiniasis. No history suggestive of trichiniasis was elicited from any patient except the first patient tested. The available evidence indicates that the trichina precipitin test remains positive for only 2 to 3 years after infection, whereas skin reactions are more persistent.[1] In contrast to this, in the present study precipitin tests were positive, but skin tests and complement fixation tests were negative. Trichiniasis is rare in children,[1] while in the present study 14 of 21 children with leukemia or lymphosarcoma had high titres. Furthermore, if the present results were due to unrecognized trichiniasis an equal distribution of positive reactions would be expected in all diagnoses.

The major import, then, of the present study is that the trichina precipitin reaction is invalid as a test for trichiniasis in patients with certain neoplastic diseases, especially Hodgkin's disease, lymphosarcoma, and acute leukemia. The data also imply the possibility that this phenomenon might be utilized in prognosis, but the investigation has not yet proceeded far enough to permit valid discussion of such a possibility.

Indications at present suggest that the positive reactions are related in some way to the neoplastic process. There has been no correlation between positive precipitin reactions and fever or eosinophilia. The infrequency of positive reactions among patients with advanced carcinoma of the breast or prostate and the frequency of positive tests among patients in good condition with Hodgkin's disease, make it unlikely that the positive reactions reflect merely debilitation. There have been no changes in titres in serial specimens which would suggest that the presence in the serum of folic acid, folic antagonists, or nitrogen mustards cause the positive reactions. Many highly positive reactions were observed in sera obtained before any treatment had been given. The addition of folic acid (pteroylglutamic acid) or 4-amino-pteroylglutamic acid (10 γ/cc) to serum having a negative precipitin reaction did not change the reaction.

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Mechanism of the reaction. The cause of these false positive trichina precipitin reactions is not known.

Summary. Patients with certain neoplastic diseases involving the reticuloendothelial system (Hodgkin's disease, lymphosarcoma and acute leukemia) exhibited a high percentage of false positive trichina precipitin reactions. Such reactions were much less frequently encountered in sera from patients with metastatic carcinomas and no positive reactions were observed in the normal controls. The false-positive reaction does not appear to be related to medication.

Received August 30, 1949. P.S.E.B.M., 1949, **72**.

Tissue Distribution and Elimination of DDD and DDT Following Oral Administration to Dogs and Rats.* (17431)

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Although the tissue distribution of ingested DDT (2,2-bis-(p-chlorophenyl)-1,1,l-trichloroethane) has been reported by several workers,[1-7] in most cases with particular reference to its storage in fat, similar data concerning its analog, DDD (2,2-bis-(p-chlorophenyl)-1, 1-dichloroethane), is limited.[5]

The increasing agricultural use of DDD (Rhothane) against insect pests infesting fruit and vegetable crops makes desirable further knowledge concerning its tissue distribution following ingestion. Although it has been demonstrated that DDD is less toxic on both an acute[8,12] and chronic[5,11,12] basis than is DDT, it has not as yet been made clear whether this difference is attributable to DDD being inherently a less toxic molecule, or whether it is due to differences in absorption, storage or excretion.

In an attempt to clarify this question of absorption, storage and excretion of DDD, the following experiments were done:

Experimental. Dogs. Ten female dogs were used, 5 receiving DDD and 5 DDT in a dose of 25 mg per kg daily except Sunday, for a maximum period of 4 weeks. The insecticides were administered orally once daily in the form of a 10% solution in corn oil in gelatin capsules at the time of the daily feeding.

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* These studies were supported by a grant from the Rohm and Haas Company, Philadelphia, Pa.

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[5] Woodard, G., Davidow, B., and Nelson, A. A., *Fed. Proc.*, 1948, **7**, 266.

[6] Smith, M. I., and Stohlman, E. F., *Pub. Health Rep.*, 1944, **59**, 984.

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[8] von Oettingen, W. F., and Sharpless, N. E., *J. Pharm. Exp. Therap.*, 1946, **88**, 400.

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TABLE I.
DDD and DDT Content of Tissues of Dogs to Which the Insecticides Had Been Administered Orally Daily for Periods of 2 and 4 Weeks.

	Insecticide content, mg per kg of tissue									
Insecticide	DDD					DDT				
Duration	2 wks		4 wks			2 wks		4 wks		
Dog No.	D-4	D-5	D-1	D-2	D-3	T-4	T-5	T-1	T-2	T-3
Liver	0	0	1.6	0	24	0	0	0	0	0
Kidney	15	4.3	13	14	15	4.9	0	14	7.2	9.6
Heart	3.8	0	11	12	13	0	3.4	4.9	8.1	3.4
Brain	0	0	3.5	4.1	5.2	1.3	0	2.5	3.9	1.2
Lung	0	0	1.9	3.9	lost	0	0	0	0	0.8
Pancreas	0	0	8.9	29	12	0	3.4	14	9.4	8.8
Spleen	0	1.7	0	12	4.7	0	0	2.2	3.6	0
Adrenal	0	0	150	0	210	0	0	83	63	62
Fat	76	270	880	360	300	29	100	910	400	200
Muscle (gastroc.)	7.6	5.2	lost	20	28	8.4	5.5	12	14	18
Skin	90	7.2	28	128	18	82	73	0	6.4	3.3
Mammary gland	—	—	—	—	—	—	—	2.2	—	21
Fecal excretion mg/kg body wt, 48 hr	0.9	4.2	3.9	2.2	3.9	lost	0.03	0.01	0.5	0.8

Twenty-four hour urine samples were taken at the end of the first, second, and fourth weeks for subsequent analysis for DDD, DDT, or their acid end-product, DDA (2,2-bis-(p-chlorophenyl) acetic acid). The total feces of each dog was collected over the 48 hour period just prior to the time of sacrifice for similar chemical analysis.

At the end of 2 weeks, 2 of the dogs in each insecticide group were sacrificed, the remaining dogs being sacrificed at the end of 4 weeks. Samples of the tissues listed in Table I were removed, frozen quickly in a dry-ice-alcohol mixture and preserved in the frozen state for subsequent analysis for DDD and DDT. In the case of 2 dogs receiving DDT that were lactating at the end of the experiment a sample of mammary gland tissue was similarly prepared. Because the presence of blood in the tissue sample appears to interfere with the analytical procedure used, as much as possible was removed (ca. 90% as determined by hematocrit) from the circulation by saline perfusion before death.

TABLE II.
DDT Content of Pooled Organs from Pups Nursing from Mothers Receiving Daily Doses of DDT Orally.

	DDT, mg per kg organ weight	
Tissue	4 pups from T-3 (2 days old)	3 pups from T-1 (2 wks old)
Liver	220	0.51
Kidney	6.3	8.3
Heart	0	10
Lung	3.5	1.3
Brain	0	0
Skin	26	12
Fat	205	0
Pancreas	149	46
Spleen	9.8	0
Muscle	8.4	5.8

During the course of the experiment, 3 of the dogs bore litters. One of the 4-week DDD dogs (D-1) bore 5 premature stillborn pups 13 days after beginning of the experiment. All of these were frozen whole for subsequent chemical analysis. On the same day, one of the 4-week DDT animals (T-1) bore 5 pups, 2 of which were sacrificed as soon after birth as they were discovered and frozen whole. The other 3 were sacrificed at the end of the experiment and the organs listed in Table II removed, pooled as to organ and frozen. Another 4-week DDT dog (T-3) bore 6 pups 2 days before the termination of the experiment. Two were killed and frozen whole before having an opportunity to nurse; the other 4 were killed at the end of the experiment and the same organs removed, pooled and frozen. Because of their small size, no attempt was made to perfuse out the blood of the pups.

Rats. The rats used in this experiment were survivors from a series of animals that had been subjected to a 2-year feeding program comparing the chronic toxicity of DDD with that of DDT. Finely ground Purina Dog Chow served as the basic diet, and into this was incorporated predetermined amounts of 5 or 10% DDD or DDT in Pyrax† to achieve the dietary concentrations listed in Table III. Only analyses of the fat were done on these animals.

All DDD, DDT and DDA determinations were done according to the Schechter-Haller method.[9,10]

Results and Discussion. Dogs. Table I shows the amounts of DDD and DDT in mg per kg of organ weight found in the tissues of the treated animals.

It will be noted that in the animals sacrificed at the end of 2 weeks the fat and the skin were the main sites of storage of both DDD and DDT. Muscle and kidney showed a measurable level of both insecticides, however, the other tissues that were studied showed either none or very small amounts.

In those animals sacrificed at the end of 4 weeks the fat, again, showed the highest content of both DDD and DDT, whereas, the skin content, which was relatively high for both insecticides at the end of 2 weeks, was found to be low in the case of the DDT animals. This lower skin content of DDT may be related to the body weight loss and resultant probable decrease in cutaneous fat content incurred by this group.

It seems that, in general DDD and DDT were deposited in fat to a similar degree. This is in agreement with the thesis of von Oettingen and Sharpless[8] that since the 2 compounds have similar solubilities in olive oil they would probably be stored to about the same degree in fat.

The other tissues in which little or no insecticide was found at the end of the 2 week period contained definite quantities of DDD or DDT at the end of 4 weeks. This is particularly noticeable in the case of the adrenal gland and is most apparent here in those animals which received DDD. This latter observation is of interest in relation to the adrenal cortical atrophy that has been described in dogs receiving DDD orally[5,13] and which has also been noted in this laboratory (unpublished).

Analysis of the pooled carcasses of the 5 stillborn pups from one of the 4-week DDD dogs (D-1) showed a content of 3.7 mg per kg DDD. Similar analyses of individual carcasses of the 2 newborn pups from dog T-1 and the 2 newborn pups from dog T-3 (both 4-week DDT dogs) gave average values of 1.3 mg per kg for the first pair and 0.04 mg per kg for the second. It was thought that the latter result was low owing to possible loss of part of the sample. These results demonstrate transfer of both DDD and DDT across the placenta.

Table II shows the amounts of DDT stored in the pooled organs of pups that were born to 2 of the dogs (T-1 and T-3) during the feeding period and nursed for the remainder of the time until the end of the experiment. It will be noted that the pups from dog T-3, which were 2 days old at the time of sacrifice, showed high levels of DDT in the liver, skin, fat and pancreas, whereas, those that had nursed for a period of 2 weeks (pups from dog T-1) showed much lower levels in these tissues, and, with the exception of heart tissue and perhaps kidney, showed lower levels in all other organs examined. This again seems indicative of a high degree of placental transfer of DDT and further suggests that the fetus is more liable to the accumulation of DDT than is the suckling offspring, despite the fact that DDT has been shown to be secreted in the milk.[3,4]

Included in Table I are data showing the amounts of the insecticides excreted in the feces in the 48 hour period just prior to sacrifice. Fecal excretion, in all cases, represented but a small proportion of the amount of the

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† A pyrophylite diluent.

[9] Ofner, R. R., and Calvery, H. O., *J. Pharm. Exp. Therap.*, 1945, **85**, 363.

[10] Schechter, M. S., and Haller, H. L., *J. Am. Chem. Soc.*, 1944, **66**, 2129.

[13] Nelson, A. A., and Woodard, G., *Fed. Proc.*, 1948, **7**, 276.

TABLE III.
Averages of Fat Analyses for DDD and DDT in Rats at the End of Two Year Feeding.

Insecticide	Dietary concentration parts per million	No. of rats	Content of DDD or DDT in body fat, mg/kg (Avg and range)
DDD	150	3	220 (180-250)
	300	7	270 (140-830)
	600	4	480 (110-950)
	900	4	350 (280-500)
	1,200	10	420 (60-790)
	1,800	5	310 (210-430)
	2,500	5	680 (320-940)
	3,750	1	1,290
DDT	150	3	2,070 (400-5,400)
	300	5	470 (240-690)
	600	1	340

insecticide administered during that period. It is apparent that DDD is uniformly excreted to a greater degree in the feces than is DDT.

In contrast to the findings of Smith *et al.*[6,12] that orally administered DDT is excreted in the urine of rabbits, it does not appear to be excreted in the urine of dogs. Neither does the dog appear to excrete DDD by the way of the kidney. However, DDA appears to be a urinary end-product common to both insecticides in dogs. Thus the average 24 hour excretion of DDD as DDA was 0.63 mg per kg body weight at the end of the first week of administration, 0.50 mg at the end of the second week, and 1.25 mg at the end of the fourth week. In the case of DDT, the DDA excretion figures were 0.13 mg, 0.12 mg, and 0.17 mg per kg body weight at the end of the same intervals. These figures also indicate that the rate of DDD excretion in the form of DDA is greater than in the case of DDT.

Rats. It is apparent from Table III that both DDD and DDT are stored in considerable amount in the fatty tissues of the rat. Among the DDD-treated animals there appears to be a tendency for deposition of DDD to increase with its increasing dietary content, although this is not entirely consistent. The number of animals and dietary levels studied using DDT was too limited to permit similar comparisons.

Conclusions. 1. DDD and DDT when administered orally are stored in the tissues of the dog, the greatest storage appearing in the fat.

2. Similarly DDD and DDT are both stored in the fat of the rat.

3. Both DDD and DDT are transported across the placental barrier in the dog.

4. In the dog, a small proportion of orally administered DDD or DDT is excreted in the feces. Of the 2, DDD appears in greater amount.

5. When administered orally to the dog, neither DDD nor DDT are excreted as such in the urine. DDA is a urinary end-product common to both DDD and DDT, appearing in greater amount following DDD administration.

We wish to extend our thanks to Dr. E. L. Stanley of the Rohm and Haas Company for performing the analytical procedures connected with this study.

Received September 1, 1949. P.S.E.B.M., 1949, **72**.

Excretion of Coproporphyrin in Rats Developing Acute Massive Hepatic Necrosis.* (17432)

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Massive hepatic necrosis in rats maintained on diets low in sulfur containing amino acids has been well described by Himsworth and Glynn.[1] This is a rapidly fatal disease which appears suddenly in a hitherto healthy animal. The lesion has been produced by feeding diets deficient in cystine and methionine where all of the nitrogen was supplied by other amino acids.[2] Hock and Fink[3] and Himsworth and Glynn[1] regularly observed hepatic necrosis on diets containing yeast as a sole source of protein. Recently Gyorgy[4] has described the regular production of this lesion when a certain type of yeast was employed as the protein source. The suddenness with which illness and death appear in such animals has been well documented[5,6] and no satisfactory biochemical means of predicting the onset of this lesion have been described. The present study was undertaken to determine whether or not an increase in the urinary excretion of coproporphyrin might herald the development of massive hepatic necrosis.

The urinary excretion of coproporphyrin in liver disease has been discussed in recent reviews.[7,8] An increase of urinary coproporphyrin is frequently regarded as a sensitive index of liver functional impairment. Clinical applications have been recently described by Watson and his associates[9] with particular reference to the distribution of the type I and type III isomer.

Glynn, Himsworth and Neuberger[2] stated that in the course of their studies on massive hepatic necrosis, the urines from certain groups of rats had been examined for porphyrins. No significant increases were found; the actual values obtained were not presented. Dent and Rimington,[6] during their attempts to produce acute hepatic necrosis, observed the rapid development of porphyrinuria in rats fed a diet which included oxidized casein. Although the diets were devoid of methionine and contained very little cystine, the rats failed to develop acute necrosis of the liver. This study, however, left unanswered the question raised above, viz: whether a heightened excretion of coproporphyrin might precede the manifestations of massive liver necrosis.

Methods. The diet employed in this experiment was identical with those employed by Gyorgy.[4]† The composition of the diet was powdered yeast,‡ 18%, cornstarch, 79% and salt mixture, 3%. Each animal received 8 g of the mixture per day. Each daily portion was supplemented with 0.5 cc of peanut oil and 2 drops of cod liver oil. Daily, each rat received 1.0 cc of a solution containing thiamine chloride 20 γ, calcium pantothenate 100 γ, pyridoxine 20 γ and riboflavin 25 γ.

The rats were maintained in individual cages. Weights were recorded once each week.

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* This investigation was supported in part by the Commission on Liver Disease, of the Army Epidemiological Board, Office of the Surgeon General, Washington, D.C.

[1] Himsworth, H. P., and Glynn, L. E., *Clin. Science*, 1944, **5**, 93.

[2] Glynn, L. E., Himsworth, H. P., and Neuberger, A., *Brit. J. Exp. Path.*, 1945, **26**, 326.

[3] Hock, A., and Fink, H., *Z. Physiol. Chem.*, 1943, **274**, 187.

[4] Gyorgy, Paul, Liver Injury, Transactions of the Eighth Conference, Josiah Macy Jr., Foundation, New York, 1949, in press.

[5] Himsworth, H. P., The Liver and Its Diseases, Oxford, 1947, Blackwell Scientific Publications.

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[7] Dobriner, K., and Rhoads, C. P., *Physiol. Rev.*, 1940, **20**, 416.

[8] Watson, C. J., and Larson, E. A., *Physiol. Rev.*, 1947, **27**, 478.

[9] Watson, C. J., Hawkinson, V., Capps, R. B., and Rappaport, E. M., *J. Clin. Invest.*, 1949, **28**, 621.

† The initial supply of yeast employed in these studies was made available to us by Dr. Gyorgy.

‡ "Kiln dried baking yeast," United Yeast Co., Ltd., 241 City Road, London E.C.I.

Food was supplied, in weighed amounts, every 2 days and water was allowed freely. Urine and feces collections were made for 48 hour periods at various intervals during the course of the study. During the collection period the rats were housed in individual plastic cages with glass rod bottoms. These cages were suspended over large glass funnels with 5 cm openings at the tapered end. Satisfactory separation of urine and feces, with little or no contamination, was accomplished by means of a specially devised glass tube attached to the lower end of the funnel. This successfully collected the urine at the side, where it drained into a tube, and permitted the feces to drop straight through into a separate container. The precaution of employing only glass and plastic in these devices was deemed necessary to prevent any contact of the urine with metal. We have noted the formation of metal porphyrin complexes during other studies when metal cages were employed, as have others also.[6] The collection periods were interrupted for 60 minutes at the end of the first 24 hours to permit feeding. An average of 3 to 5 cc of clear urine was obtained during each 24 hour period from rats weighing 40 to 90 g each. The sides of the funnels were washed down with 10 cc of water at the end of each collection period. The urine plus the washings were then refrigerated. The coproporphyrin analyses were carried out on the total 48 hour urine and fecal collections within 12 hours of the final collection time.

The estimation of the fecal coproporphyrin was conducted according to the method of Schwartz and Zagaria.[10] This method was slightly modified to permit its use in the analyses of the urine samples. During the analyses of both urine and feces, the initial 5% HCl samples were each diluted to 0.4% and extracted exhaustively with chloroform to remove protoporphyrin and deuteroporphyrin. The completeness of the separation of the coproporphyrin from these two porphyrins was checked by examining the chloroform washings under the ultra-violet light. The coproporphyrin was quantitated by readings in the fluorophotometer (Lumetron) using a solution of 1 μg of coproporphyrin per 1 cc as the standard reference.

Estimation of the coproporphyrin isomer content was performed by pooling the 1% HCl fractions from 3 urine or 3 fecal collections. Procedure B of the method described by Schwartz and his co-workers[11] was employed for the determination of the percentage of isomer I and III.

Results. Experimental observations were made on 21 rats whose initial weights ranged from 36 to 88 g. The data obtained is summarized in Table I. Fourten of the rats died spontaneously between the 30th and 125th day. Each of these animals exhibited the characteristic gross and microscopic findings of acute massive hepatic necrosis as described and illustrated by Himsworth.[5] Representative specimens are shown in Fig. 1 and 2. In most instances the rats were found dead; in a few instances the abrupt change from an apparently normal healthy status to a moribund one was observed in a matter of a few hours. Occasionally convulsions preceded death. No attempt was made to administer glucose solution, though hypoglycemia has not been excluded as a possible cause of the terminal convulsive seizures.

The remaining 7 animals were sacrificed after 41, 51 and 59 days as shown in Table I. In no instance in this group was it possible to detect gross or microscopic changes in the liver.

The data relating to the urinary and fecal coproporphyrin excretion is shown in Table I. Because of other experiments it was not possible to house the animals in the special collecting chambers all of the time; random samplings were therefore obtained. Control values for 48 hour collections of urine and feces in 6 rats maintained on a diet of fox chow just before the start of this experiment are shown in Table I. An additional 6 rats of similar weight and maintained on a similar control diet revealed urinary values of 4 to 20 γ of coproporphyrin and fecal values of

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[10] Schwartz, S., and Zagaria, R., Metallurgical Laboratory Report CH-3600, Chicago, 1946.

[11] Schwartz, S., Hawkinson, V., Cohen, S., and Watson, C. J., *J.B.C.*, 1947, **168**, 133.

TABLE I. Urine and Fecal Coproporphyrin Excretion in μg per 48 hr in Rats Maintained on an 18% Yeast Diet.

Rat No.	Sex	Material studied	Control† values	Day of experiment 11	19	23	31	35	39	42	44	49	77	Day terminated	Initial wt (g)	Final wt (g)	Outcome	Hist. find. (liver)
52	F	U			7									30	40	42	D	Massive necrosis
		F			5													
55	M	U			8		14							38	42	60	D	”
		F			3		5											
53	M	U			15		17							41	36	48	D	”
		F			4		7											
54	M	U			4		12							41	36	53	D	”
		F			5		4											
56	M	U		22		11		19	6*					41	70	57	D	”
		F		9		2		3	2*									
43	M	U	16						19					44	73	78	D	”
		F	9						8									
63	M	U						25			9	6		58	58	71	D	”
		F						3			5	5						
44	M	U	16						25					59	66	82	D	”
		F	11						5									
41	M	U	15						13					59	71	80	D	”
		F	5						5									
60	M	U		21		19		22		12		15		59	60	86	D	”
		F		6		8		4		7		6						
65	F	U						19			12	23		62	63	75	D	”
		F						4			2	4						
40	M	U	18						20					74	74	86	D	”
		F	9						5									
45	M	U	18										16	125	78	88	D	”
		F	6						5				13					
42	M	U	10						18				17	79	88	90	D	”
		F	8						9				18					
50	M	U			18		13							41	40	65	S	Normal
		F			7		5											
51	M	U			5		13							41	40	44	S	”
		F			10		9											
57	M	U		34		19		20		17		13		51	65	69	S	”
		F		4		5		6		4		3						
58	M	U		18		11		18		15		15		51	60	58	S	”
		F		9		4		4		5		6						
59	M	U		22		23		14		10		13		51	63	66	S	”
		F		5		4		4		4		5						
64	M	U						14			16	23		59	62	74	S	”
		F						3			3	4						
61	M	U		24		8		13		19		6		81	60	69	S	”
		F		9		2		2		3		2						

U—Urine.
F—Feces.

D—Spontaneous death.
S—Sacrificed.

* 24 hr collection; animal died during second 24 hr period.

† Animals maintained on stock diet (Fox Chow).

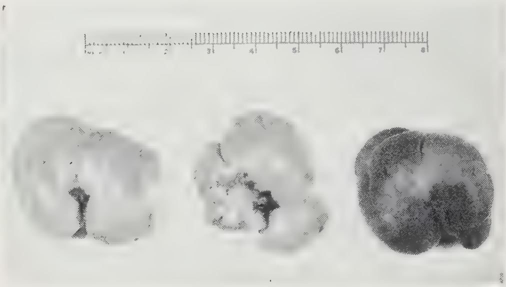

FIG. 1.
Gross appearance of rat liver showing massive hepatic necrosis as contrasted with unaffected experimental animal and a normal control, photographed after 4 hours fixation in formalin. The dark areas in the right hand specimen represent extensive areas of hemorrhagic necrosis (rat 56, death on 41st day). The center specimen (rat 51, sacrificed on 41st day) revealed no evidence of necrosis. The specimen to the left was taken from a normal animal of similar weight.

6 to 13 γ for 48 hour periods.

It is apparent from these data that no significant increases in the urinary excretion of coproporphyrin occurred in these animals. Rat 56 died suddenly during the second 24 hours of the collection period. The urinary and fecal values obtained during the first period (Table I) were not elevated. Estimation of the coproporphyrin isomer content was made in pooled samples of the material under study. Eighty to 90% of the coproporphyrin present behaved as the type III isomer by the differential precipitation "fluorescence quenching" technic.[11] A similar finding was noted in urine and fecal samples from the normal rats.

As has also been noted by others,[2,6] a few of the animals in this study exhibited traces of bilirubin in the urine one or two days before death but this was not common or constant. No increases in urobilinogen were observed; urinary amino acid excretion was not studied. A few specimens of urine were examined for the presence of homogentisic acid with negative results. The presence of this or a closely related substance in the urine of rats developing hepatic necrosis was observed by Glynn, Himsworth and Neuberger.[2] This occurred in rats fed an amino acid diet deficient in cystine and methionine. The question as to whether or not not renal damage might have prevented the excretion of pathological amounts of coproporphyrin may be raised. Histologically the kidneys of these animals appeared normal.

The excretion of excessive amounts of coproporphyrin III in the urine of humans is a

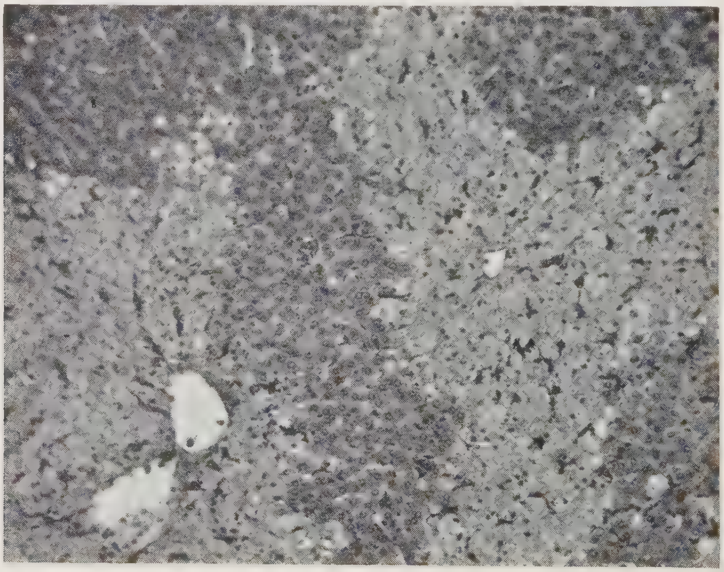

FIG. 2.
Photomicrograph, liver, rat 56, areas of necrosis in contrast with normal appearing liver cells, are shown in this section. In most sections, the areas of necrosis are more extensive. (H and E × 125).

frequent accompaniment of chemical or heavy metal toxicity, with or without evidence of hepatic functional impairment.[8,9] The failure to observe excesses in this experiment is in better agreement with the belief that the acute massive hepatic necrosis is not due to a chemical or poison. The histologic appearance of these livers suggests that the lesion develops very rapidly since there appears to be little evidence of antecedent damage as judged by infiltration or any attempt at a reparative process.

Summary. 1. The urinary and fecal excretion of coproporphyrin was studied at varying intervals in 21 young rats maintained on a diet containing 18% yeast as a source of protein.

2. Fourteen of the rats died suddenly between the 30th and the 125th day of acute massive hepatic necrosis. The gross and microscopic findings were identical with those described by Himsworth as characteristic of this lesion.

3. No significant increase in the excretion of coproporphyrin was observed during the course of these experiments. This is believed to minimize the likelihood of chemical or metal toxicity as the cause of the necrosis.

Received September 1, 1949. P.S.E.B.M., 1949, **72**.

Comparative Effect of Aureomycin and Chloromycetin on Psittacosis Infection in Chick Embryos.* (17433)

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Aureomycin[1] and chloromycetin[2,3] are antibiotics which have a similar spectrum of activity that includes many bacteria, rickettsias and viruses of the psittacosis-lymphogranuloma venereum group. With respect to psittacosis, Wong and Cox[4] found aureomycin to be highly effective against infection of embryonated hen's eggs with the 6BC strain, and Smadel and Jackson,[5] using the same strain, demonstrated similar beneficial effects from chloromycetin. The conditions of the experiments of these 2 groups of workers, however, were not entirely comparable. It seemed of interest, therefore, to make a direct comparison of the effects of these 2 agents under more nearly identical conditions. Such a comparison is reported in this paper.

Materials and methods. A uniform source of virus for the present experiments was obtained in the following manner: A stock yolk-sac suspension of the 6BC strain of psittacosis virus was injected into the yolk sac of a number of 7-day chick embryos which were then incubated at 35°C. On the 5th day, when deaths began to occur, the yolk sacs were harvested and pooled. An equal volume of broth was added and the mixture homogenized in a Waring blendor. The resulting 50% yolk sac suspension which, on culture, was found to be free of bacterial contamination, was distributed in sealed pyrex ampoules, quick-frozen and stored at −70°C. The LD_{50} of this suspension was about 10^{-8} throughout these experiments. Yolk sac smears of infected embryos stained by Macchiavello's technic showed numerous characteristic elementary bodies.

A fresh solution of crystalline aureomycin

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* Aided by a grant from the United States Public Health Service.

† Research Fellow in Virus Diseases, National Research Council.

[1] Duggar, B. M., and others, *Ann. N. Y. Acad. Sci.*, 1948, **51**, 175.

[2] Gottlieb, D., Bhattacharyya, P. K., Anderson, H. W., and Carter, H. E., *J. Bact.*, 1948, **55**, 409.

[3] Smith, R. M., *et al.*, *J. Bact.*, 1948, **55**, 425.

[4] Wong, S. C., and Cox, H. R., *Ann. N. Y. Acad. Sci.*, 1948, **51**, 290.

[5] Smadel, J. E., and Jackson, E. B., *Science*, 1947, **106**, 418.

hydrochloride[‡] was prepared just prior to use in each experiment. A solution containing 8 mg of crystalline chloroamphenicol[§] (chloromycetin) per ml was made, also in broth, but this was heated to 100°C for 15 minutes and and then stored at 4°C for use in 2 or 3 experiments. The chloromycetin remained in solution throughout this study and retained its potency when assayed against a standard test organism. Both antibiotics were diluted for use so that the desired dose was contained in 0.25 ml of broth.

All inoculations were made into the yolk sac of 7-day-old embryonated eggs. In each experiment, groups of 6 to 12 eggs per dose were each injected with aureomycin in amounts ranging from 1.0 mg to 0.0625 mg and comparable groups were inoculated with similar amounts of chloromycetin. These eggs were then inoculated with a 10^{-3} dilution of the virus suspension. A titration of the virus was included in each experiment and this inoculum was regularly found to contain about $10^5 LD_{50}$. The time elapsed between injections of drug and virus was about 45 minutes. Uninfected drug controls and untreated virus-infected controls were each given a second injection of plain broth. The eggs were incubated at 35°C; they were candled and the deaths noted daily for 12 days, after which the experiment was terminated. Deaths occurring before the third day were considered traumatic. The dose of drugs that protected 50% of embryos (PD_{50}), and the LD_{50} were calculated by the method of Reed and Muench.[6] This method was also used to calculate the day when 50% of the embryos died (DD_{50}).[||]

Results. Similar results were obtained in 6 separate experiments. The data for all 6 experiments have been combined in Table I.

TABLE I. Effect of Aureomycin and Chloromycetin on Psittacosis (6BC) Infection of Chick Embryos. Summation of 6 experiments.

Antibiotic	Dose, mg	No. of embryos infected*	No. of embryos surviving: Days after infection 3	4	5	6	7	8	9	10	11	12	DD_{50}†	MPL,‡ days	% survivals: Days after infection 8	10	12
Aureomycin	1.0	22	22	21	21	20	19	19	15	13	12	10	11.8	7.2	86	59	45
,,	.5	54	54	53	52	50	47	38	37	30	27	21	11.6	7.0	70	56	39
,,	.25	56	56	55	54	54	51	37	24	15	9	6	9.7	5.1	66	27	11
,,	.125	57	57	56	55	46	41	22	10	5	0		8.1	3.5	39	9	0
,,	.0625	35	35	35	33	23	15	5	1	0			7.0	2.4	14	0	0
Chloromycetin	1.0	43	43	42	39	36	34	24	19	11	6	3	9.5	4.9	56	26	7
,,	.5	50	50	50	49	48	37	26	10	3	0		8.3	3.7	52	6	0
,,	.25	53	53	52	47	37	17	3	1	0			6.7	2.1	6	0	0
,,	.125	52	52	52	44	21	2	0					5.9	1.3	0	0	0
,,	.0625	12	12	11	7	0							5.1	0.5	0	0	0
Broth		57	57	51	10	0							4.6		0	0	0

Infecting dose per embryo = 85,000 LD_{50}, calculated on 8th day. (See also Table II).

* Number which survived more than 2 days.

† Day when 50% of embryos died (calculated).

‡ Mean prolongation of life. (DD_{50} for each dose minus DD_{50} for unprotected controls).

[‡] Supplied in sterile vials by the Lederle Laboratories.

[§] Furnished by the Department of Clinical Investigation, Parke, Davis and Co.

[6] Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

[||] This method was chosen in preference to the usual calculation for "mean day of death" in order to avoid the error introduced by the embryos which survived at the end of the experiment.

TABLE II.
Protective Dose.

Days after infection	PD_{50} (mg)		LD_{50} of infecting dose*
	Aureomycin	Chloromycetin	
7	.09	0.36	1.8×10^4
8	.20	0.61	8.5×10^4
9	.31	>1	3.1×10^5
10	.42	>1	6.2×10^5
11	.51	>1	9.6×10^5
12	.62	>1	$>10^6$

* The number of LD_{50} contained in the infecting dose will vary according to the time of observation; and the figures in this column indicate the apparent number of LD_{50} on the days indicated in the first column.

The infected and unprotected control embryos all died between the 4th and 6th days. The DD_{50} for this group was 4.6 days. In the treated groups, the DD_{50} varied directly with the dose.

The beneficial effects of the 2 antibiotics may be compared on the basis of the mean prolongation of life (MPL) which, for convenience may be considered as the difference between the DD_{50} of any treated group and that of the control group. The MPL for the aureomycin-treated eggs was greater than that of the chloromycetin-treated groups at each dosage level. The MPL for the groups of embryos receiving doses of 0.5 and 0.25 mg of aureomycin was 3.3 and 3.0 days longer, respectively, than for those receiving the corresponding doses of chloromycetin. At each of the other dosage levels the MPL was about 2 days longer in the aureomycin treated groups.

The greater protective effect of aureomycin as compared with chloromycetin is also strikingly apparent from the percent of survivors among the groups of embryos treated with various doses of antibiotics. This was calculated for each dose at different times after infection and the figures for the 8th, 10th and 12th days are shown in Table I.

A more quantitative estimation of the relative effectiveness of the 2 drugs may be arrived at by comparing the dose required to protect 50% of the embryos (PD_{50}) for any given number of days after infection. The result is shown in Table II for days 7 to 12, inclusive. In the case of aureomycin, it is seen that the PD_{50} increased at a steady rate of approximately 0.1 mg per day during this period. The increase for chloromycetin was at a much more rapid rate between the 7th and 9th days, after which time the PD_{50} of this agent exceeded 1.0 mg, the largest dose used in these experiments. The PD_{50} of aureomycin on days 7, 8 and 9 was 0.09, 0.20 and 0.31 mg, respectively, as compared with 0.36, 0.61 and more than 1.0 mg for chloromycetin on the corresponding days. Thus on these 3 days, aureomycin was from 3 to 4 times as active as chloromycetin, weight for weight, in protecting the embryos against the infecting dose of virus used. A similar advantage in favor of aureomycin was noted after the 9th day, but this was not quantitated since the PD_{50} for chloromycetin was greater than 1.0 mg, the largest dose used. [The infecting dose on the basis of the LD_{50} calculated for each of these days, is also shown in Table II.]

Discussion. The results indicate that the life of chick embryos infected with the 6BC strain of psittacosis virus was prolonged for an average of from 2 to more than 3 days longer by aureomycin than they were by the same dose of chloromycetin. Also, the dose of chloromycetin required to protect 50% of infected embryos for 7 to 9 days was 3 to 4 times as great as the dose of aureomycin required to produce the same effect. Since the molecular weight of aureomycin is 508[7] as compared with 323 for chloromycetin,[8] the PD_{50} of the former is more than 5 times that of the latter on a molecular basis.

Statistically, the observed differences were highly significant.

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[7] Broschard, R. W., *et al.*, *Science*, 1949, **109**, 199.

[8] Controulis, J., Rebstock, M. C., and Crooks, H. M., Jr., *J. Am. Chem. Soc.*, 1949, in press.

These findings were rather unexpected in view of the rapid deterioration of aureomycin *in vitro* at neutral or alkaline pH, and at the temperature employed.[9] Other factors in the embryo, however may affect the stability of the two agents and some of these factors are now under study.

Conclusions. Both aureomycin and chloromycetin prolong the life of chick embryos infected with the 6BC strain of psittacosis virus. There is a direct relation between the dose of antibiotic and the prolongation of life of the embryos, the infecting dose being kept constant. With equal doses of the antibiotics, aureomycin prolonged the life of the embryos for an average of from 2 to more than 3 days longer than chloromycetin, depending on the dose used.

On a weight basis, aureomycin was more than 3 times as effective in protecting chick embryos against the dose of virus that was used in this study. On a molecular basis the aureomycin was more than 5 times as effective as chloromycetin in this infection.

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[9] Paine, T. F., Jr., Collins, H. S., and Finland, M., *J. Bact.*, 1948, **56**, 489.

Received September 6, 1949. P.S.E.B.M., 1949, **72**.

Hypotension Associated with Nutritive Failure.* (17434)

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During the 12 years we have been studying persons with nutritive failure in a large Nutrition Clinic, we have learned that there is a great variation in blood pressure among such persons. Readings range from those which are too high to be obtained on an ordinary sphygmomanometer to those which are extremely low. In general, however, we have found that in the person with nutritive failure the blood pressure usually is below normal and that it tends to rise slowly as the nutritive failure is corrected. Even in patients with both hypertension and nutritive failure, we have observed that the blood pressure tends to increase slightly when the nutritive failure is corrected and that they lead a more energetic life.

It occurred to us that it would be worthwhile to determine the effect of desoxycorticosterone acetate on blood pressure. Since it has been known for some time that the development of edema, hypertension, and cardiac failure are the chief dangers associated with overdosage of desoxycorticosterone acetate,[1-4] we selected for study 3 persons who had been under observation in our Clinic for many years and who had every evidence of a properly functioning cardiovascular system except that the blood pressure was below normal. Each morning for a month these patients came to the Clinic early for special consideration and study. After they became accustomed to this routine, their blood pressures were taken under resting conditions each morning at 20 minute intervals over a period of 2 hours for 2 days. Then they were given intramuscular injections of sterile saline and the blood pressure readings were taken as before. This procedure was continued for 4 days. When no systolic reading above 100 and no diastolic reading above 65 was obtained, the patients were considered

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* This study was aided by grants from the Martha Leland Sherwin Fund and the Upjohn Company.

The desoxycorticosterone acetate used in this study was supplied by Dr. Kenneth W. Thompson of Organon, Inc.

† Clayton Foundation Fellow.

[1] Ferrebee, J. W., Ragan, C., Atchley, D. W., and Loeb, R. F., *J.A.M.A.*, 1939, **113**, 1725.

[2] Grollman, Arthur, Essentials of Endocrinology, Philadelphia, J. B. Lippincott Company, 1941, p. 317.

[3] Soffer, Louis J., Diseases of the Adrenals, Philadelphia, Lea and Febiger, 1948, p. 162.

[4] Hartman, Frank A., and Brownell, Katharine A., The Adrenal Gland, Philadelphia, Lea and Febiger, 1949, p. 223.

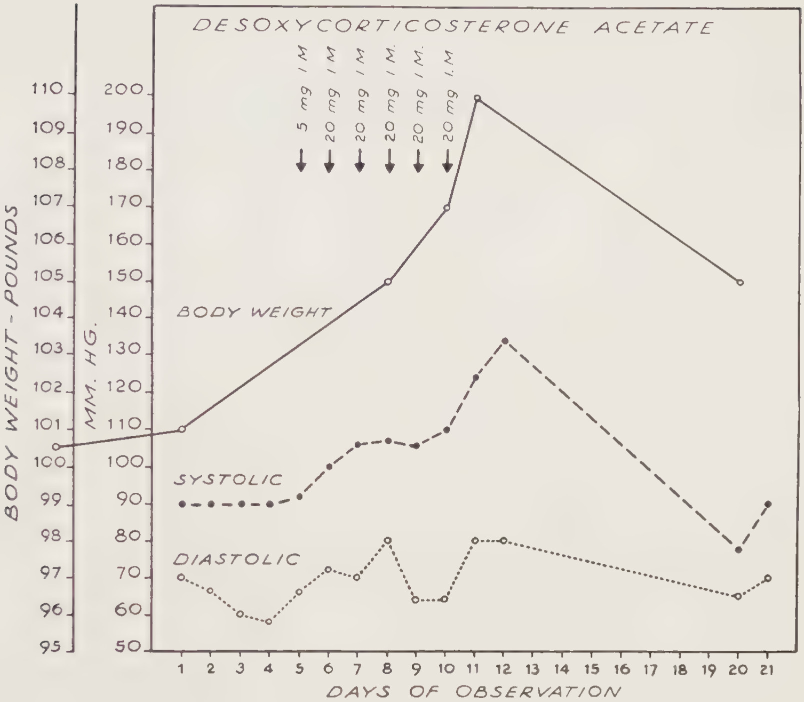

Fig. 1.

Shows the effect of the injection of 105 mg of desoxycorticosterone acetate over a period of 6 days. The diastolic and systolic blood pressure and the body weight increased. Note the drop in the blood pressure determinations and the body weight following cessation of administration of the desoxycorticosterone acetate. The increase in body weight was associated with swelling of the ankles which decreased as soon as the material was discontinued.

to be in a suitable condition to initiate injections of desoxycorticosterone acetate. It was injected intramuscularly early in the morning and a series of blood pressures was taken as before. The first day each of the 3 patients was given 5 mg of desoxycorticosterone acetate intramuscularly; the second day 1 of the patients was given 5 mg and 2 were given 20 mg each; the third day 1 was given 10 mg and 2 were given 20 mg each. On the fourth, fifth, and sixth days the dosage in each case was as shown in Fig. 1. On the third day following the injection of the initial dose of desoxycorticosterone acetate, the patients began to complain of tightness of the skin over the ankles. Fig. 1, which is representative of the 3 cases, illustrates the effect of desoxycorticosterone acetate on the systolic and diastolic pressures and on the body weight.

Summary and conclusions. Following the administration of desoxycorticosterone acetate to persons with nutritive failure and hypotension, there was a prompt increase in blood pressure readings and in body weight (the increase in body weight is associated with retention of sodium in the tissues and this problem is being studied further). When the administration of this substance was discontinued, the blood pressure readings promptly returned to normal. During the first week after the administration of desoxycorticosterone acetate was begun, one patient stated that she had great symptomatic improvement; one complained of severe headaches, and she developed a "moon-faced" appearance which gradually disappeared when the injections were discontinued; the third patient noticed only swelling of the ankles. While our findings are of general interest, we do not recommend the use of desoxycorticosterone acetate as a therapeutic agent for hypertension.

Received September 7, 1949. P.S.E.B.M., 1949, 72.

Effect of Adrenal Cortex Extract on Blood Cells of the Embryonic Chick. (17435)

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In a previous paper,[1] it was shown that a transient lymphopenia and concomitant leukocytosis occurs in the adult fowl after a single injection of adrenal cortical extract, thus resembling mammalian blood cell changes reported by others.[2]

The present study was undertaken to determine the response of embryonic blood cells to adrenal cortex extract (ACE). In addition, other effects of the extract were observed in the hope that we might add to the meagre information known on the role of the adrenal cortex in the embryo.[3]

Method. Single Comb White Leghorn chick embryos were used. Sterile 0.85% saline (control) and aqueous adrenal cortex extract* were injected into the allantoic cavity of embryos incubated 13, 14, 15 days, the dosage varying from 0.2 to 0.6 cc. Each embryo received only a single injection. One embryo series was bled from the vitelline vein[4] 24 hours after injection, while the second series was injected but allowed to hatch. In the first group, each embryo was bled once, and after bleeding was weighed to the nearest tenth gram, with extra-embryonic material removed.

Blood was diluted to the 101 mark in a certified RBC diluting pipette, the diluent[5] consisting of 25 mg of neutral red solution added to 100 cc of 0.85% saline. Total absolute counts per cubic millimeter (mm^3) of erythrocytes and leukocytes combined, were made in a Neubauer Brightline Counting Chamber.

Blood smears were stained with Wright's solution and 200 fields were examined on each smear. A Whipple disc was employed to determine the average number of cells in a field. Using a simple proportion, the actual count of a particular cell on a smear was converted to an absolute count:

$$\frac{\text{Smear count}}{\text{Avg No. cells in field} \times 200} =$$

$$\frac{\times \ (\text{Absol. No. cells/mm}^3}{\text{Total absol. count/mm}^3}$$

In the second series, the hatched chicks were bled on alternate days from the ulnar vein, over a period of one month after hatching, and the weight of every specimen taken after each bleeding. Smears were stained in the usual manner and differential white blood cell counts were made by counting 200 leukocytes in equally distributed sections of each slide.

Results. The injection of 0.3 cc of ACE had no apparent effect on the number of lymphoid cells in the peripheral blood of chick embryos of 14 to 16 days incubation. However, the hormone had a potent influence on the polymorph leukocyte number (Table I). Applying the chi-square test[6] to the frequency distribution of lymphoid cells (lymphocytes and hemocytoblasts[7]) and polymorph leukocytes, showed that chance accounted for the lymphocyte distribution, whereas the presence of the cortical extract significantly affected the distribution of the leukocytes.

The blood cell count means are shown in Table II. A statistical analysis of the difference between the means of the saline and ACE groups was not undertaken because of

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[2] White, A., and Dougherty, T. F., *Ann. N. Y. Acad. Sci.*, 1946, **46**, 859.

[3] Landauer, W., *Endocrinology*, 1947, **41**, 489.

* The extract was furnished through the courtesy of Dr. David Klein of the Wilson Laboratories.

[4] Zifferblatt, A. N., and Seelaus, H. K., *Anat. Rec.*, 1931, **48**, 367.

[5] Forkner, C. E., *J. Exp. Med.*, 1929, **50**, 121.

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[7] Fennell, R. A., *J. Agric. Res.*, 1947, **74**, 217.

TABLE I.
Effect of 0.3 cc ACE on Lymphocytes and Polymorph Leukocytes of Embryonic Chicks Injected on the 13th, 14th, and 15th Day of Incubation and Bled 24 Hours Later. Application of the Chi-Square Test.

Class mark (No. cells/mm^3)	Observed frequency (f) Sal.	Observed frequency (f) ACE	Expected frequency (f′) Sal.	Expected frequency (f′) ACE	(χ^2) $(f-f')^2/f'$ Sal.	(χ^2) $(f-f')^2/f'$ ACE
		Lymphocytes				
0 to 251	24	30	25.1	28.9	.048	.042
252 to 751	23	26	22.8	26.2	.002	.002
752 to 1251	9	10	8.9	10.1	.001	.001
1252 to 2251	12	10	10.2	11.8	.320	.271
2252 to 9751	7	10	7.9	9.1	.102	.089
Totals	75	86	74.9	86.1		.878*
		Polymorphonuclear leukocytes				
0 to 101	48	39	40.4	46.6	1.420	1.230
102 to 301	9	10	8.8	10.2	.004	.004
302 to 2101	8	26	15.8	18.2	3.850	3.340
Totals	65	75	65.0	75.0		9.848†

* $\chi^2 = .878$, 4 degrees freedom: P = .95 or chance accounted for 95% of the observed frequency distribution.

† $\chi^2 = 9.848$, 2 degrees freedom: P = .01 or ACE accounted for 99% of the observed frequency distribution.

TABLE II.
Effect of Single Injection of ACE and Saline (0.3 cc each) on the Blood Count of Embryos, Bled on the 14th, 15th, and 16th Day of Incubation (Injected 24 Hours Previously). Mean Values.

Cell (No./mm^3)	14 days Sal.	14 days ACE	15 days Sal.	15 days ACE	16 days Sal.	16 days ACE
Lymphocyte	1,978	1,791	1,197	924	619	729
S.D.*	2,800	1,902	708	694	606	647
No. embryo	(27)	(34)	(16)	(18)	(32)	(34)
Polymorphs	106	309	108	319	152	416
S.D.	138	421	282	302	174	484
No. embryo	(17)	(23)	(16)	(18)	(32)	(34)
RBC† × 10^3	1,926	1,816	1,844	1,889	1,961	1,845
S.D.	268	297	187	229	334	328
No. embryo	(30)	(36)	(16)	(18)	(32)	(35)

* S.D. $= \frac{1}{N}\sqrt{N\,\Sigma(x^2) - \Sigma(x^2)}$, where N is the number of embryos, and x is the individual value of the mean.

† is equivalent to number of total cells in counting chamber.

the large standard deviation (S.D.) and the skewness of the distribution of the individual counts. The erythrocytes do not seem to have been affected by ACE since the mean total absolute count, consisting mainly of erythrocytes, was approximately the same in both treated groups during the incubation period considered.

Table III shows that the hormone did not affect embryo weight significantly, although a slight trend toward a weight loss was present in the 14 day hormone-injected embryos.

The presence of ACE depressed the hatchability rate of embryos in the second series (injected and allowed to hatch). Out of 154 saline-treated embryos, 82.4% hatched, while 68.4% of 149 hormone-injected embryos hatched. The critical ratio of the per cent

TABLE III.

Effect of Single Injections of ACE and Saline (0.3 cc each) on the Wet Weight of Embryos Weighed 24 Hr After Injection on the 14th, 15th, and 16th Day of Incubation. Mean Values.

	14 days		15 days		16 days	
	Sal.	ACE	Sal.	ACE	Sal.	ACE
No. embryos	(47)	(60)	(16)	(18)	(38)	(38)
Wet weight (g)	8.1	6.3	12.3	11.1	13.1	13.1
S.D.	1.0	1.2	0.9	1.0	1.1	1.5
S.E.*	1.53		1.33		1.85	
t	1.22		.90		.56	

* S.E. (standard error) $= \sqrt{\sigma M_1^2 - \sigma M_2^2}$, where σ is the standard deviation of M_1 and M_2, the means. $t = \frac{M_1 - M^2}{S.E.}$, t = 2.2 is significant.

difference was 2.86 which is statistically significant, since a ratio of 2.57 is equivalent to 99% confidence.

No discernible differences in the blood smears or weights of the hatched chicks between the 2 treated groups were observed. An apparent greater degree of barren skin area under the wings and abdomen and whiter down feathers were noticed in the ACE hatched chicks as compared to the control group.

Discussion. In the present study no change could be detected in the number of lymphoid cells after administration of adrenal cortical extract. However, there was a significant leukocytosis upon hormonal injection.

Failure to detect a transient lymphopenia is not in agreement with previous work on the adult fowl and mammals,[1,2] but we do not believe that such a conclusion is warranted in the present study. The relatively small number of lymphocytes on a smear and the resemblance of their precursors (hemocytoblast) to certain primitive erythrocytic cells,[7] renders the lymphocyte count less reliable than the counts of the more readily identifiable polymorphs. Furthermore, the large individual variation in lymphocyte numbers in the embryos would tend to obscure the hormonal effect.

Our failure to observe a lymphopenia after ACE injection might also be attributed to the length of time (24 hours) elapsing between injection of the hormone and removal of blood samples. Lymphopenia in adult mammals and fowl occurs at about 3-6 hours post injection. No lymphopenia was observed in 15 embryos from which blood was removed at intervals of 3, 6, or 9 hours after injection of ACE. The period of 24 hours was selected for most of the specimens in order to allow for a greater degree of absorption from the chorioallantois, although the possibility remains that a shorter period might have been more effective.

Erythrocytes showed no response to ACE which corresponds with results in the adult fowl.[1] Variation of the dose from 0.2 to 0.6 cc had no apparent effect on the general embryonic blood picture.

The weight loss of embryos injected with adrenal cortical extract, although not statistically significant, taken together with the definite decrease in hatchability, suggests that the hormone retarded embryonic metabolism. This may have been due to the extract's toxicity or perhaps to its protein catabolic action.[8] Landauer,[3] reported that the injection of either 0.02 or 0.05 cc of ACE into 0 or 120 hour chick embryos produced a very marked retardation of growth and, with the larger amount of hormone, none of the embryos completed development. The use of 11-desoxycorticosterone or other steroids having no effect on protein metabolism,[8] may reveal whether ACE is toxic, as it seems to be, to the embryonic organism.

Thiourea injected into chick embryos has been reported[9] to inhibit the thyroid, lower body weight and produce a feather syndrome similar to the one here reported. This suggests that ACE may act indirectly on the embryo by depressing the thyroid to the extent of interfering with local feather and general embryonic metabolism.

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Summary. 1. The blood picture of 14, 15 and 16 day White Leghorn chick embryos injected into the allantois with aqueous adrenal cortex extract, shows no significant change in the number of lymphocytes but a marked absolute polymorph leukocytosis, 24 hours after injection. The erythrocytes are not affected by the extract.

2. Large individual variation in the lymphocyte count and greater difficulty involved in their identification may have obscured the effect of the hormonal preparation on the lymphocyte count.

3. ACE treatment induced a tendency (not statistically significant) toward weight loss in the 14 day embryos, a significant decrease in the hatchability rate, and an apparent defective feather development. However the blood picture and weight in comparison with the controls was normal after hatching.

Received September 7, 1949. P.S.E.B.M., 1949, **72**.

Glycerophosphatases of the Normal and Tumorous Frog Kidney. (17436)

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Lucké[1] described a neoplastic growth in the kidneys of the leopard frog, *Rana pipiens* Schreber (Fig. 1), which was manifested sometimes as a circumscribed adenoma and, at other times, as an adenocarcinoma with considerable infiltrative and destructive activities. The incidence, as reported by Lucké[1] and corroborated by the present studies, may be as much as 2% among both sexes.

It has been demonstrated that the alkaline phosphatase of the kidneys of many animals shows considerable activity.[2,3] Since it is further known that the phosphatase content of an organ is frequently altered under various pathological conditions[4,5] and especially in neoplasia[6] it was felt that studies of the alkaline and acid phosphatase systems might add to our knowledge of the physiology of the normal and pathological frog kidney.

Experimental procedure. Kidneys, both normal and tumorous were treated with a modified Gomori method recommended by Kabat and Furth.[7] Pieces of fresh kidney were fixed in cold acetone at 0°C for 24 hours and embedded in paraffin-Bayberry wax. Sections cut at 5 μ were mounted, deparaffinized and transferred to the buffered sodium glycerophosphate mixture and incubated for 8 hours at 37°C. Control sections were maintained. Some sections were stained in the usual way with Harris' hematoxylin and eosin; others were stained with safranin.

Tissues which were to be compared, namely, normal, neoplastic and calcium controlled sections were incubated and stained simultaneously.

Acid phosphatase was demonstrated by the Wolf, Kabat, and Newman[8] modification of Gomori's original method.[9]

Stewart[10] has pointed out that in cross sec-

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[3] ——— ———, *Physiol. Rev.*, 1932, **12**, 384.

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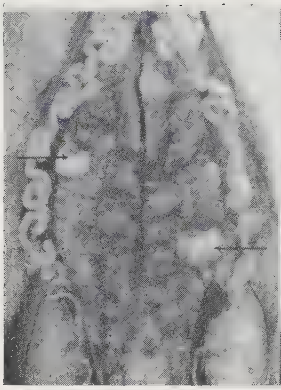

FIG. 1

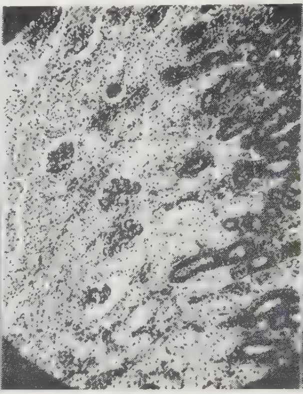

FIG. 2

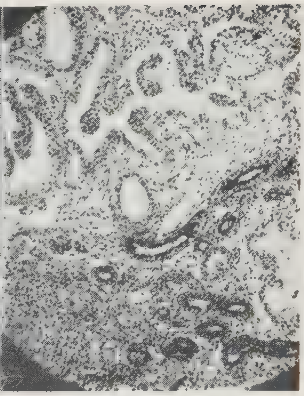

FIG. 3

FIG. 1. Bilateral tumor (T34) of a male *Rana pipiens* fixed in Kaiserling fluid. × 10.

FIG. 2. Alkaline phosphatase activity in the normal frog kidney (N6). The proximal convoluted tubules (at the right) show a +++ reaction, the glomeruli a +++ reaction, and the distal tubules (at the left) are negative. Safranin stained. × 110.

FIG. 3. Alkaline phosphatase activity in the tumorous frog kidney (T25). The gland-like tubules of the tumorous (upper) portion show a negative phosphatase reaction, whereas the tubules of the adjacent non-tumorous portion (lower quarter) indicate a reduced enzyme activity from that seen in the normal (Fig. 2). Safranin stained. × 110.

tion the frog's kidney is approximately hemicircular and may conveniently be divided into 3 crescent-shaped segments—the dorsal, intermediate and ventral zones. The proximal convolutions are quite uniformly restricted to the dorsal zone in which is located practically the entire system of collecting tubules. The distal convolutions were mainly found to lie in the ventral zone. In the central zone are grouped the glomeruli along with the transitions of the proximal to distal convolutions. Our results were tabulated using this description of the locations of the various kidney segments.

The intensity of the phosphatase reaction was graded by a system of pulses based on the size, distribution and density of granules in several sections: ++++ maximum reaction, *i.e.,* one in which the phosphatase deposition was so dense that the nuclear outline was just discernible, +++ large amount, ++ moderate, + slight, – negative.

Results. 1. *Sites of alkaline phosphatase activity in normal and tumorous kidney.* Alkaline phosphatase was consistently found to be heavily concentrated at the luminal borders of the proximal tubules and in the collecting ducts. A fair amount of the enzyme was also found in the glomeruli. However, the glomerular capsule and the distal convolutions were always found to be negative (Fig. 2).

There was no difference in the alkaline phosphatase picture between the male and female kidneys, neither did there appear any preferential staining for any portion of the same kidney, *i.e.,* serial sections of normal kidneys excluded the possibility of local variations in phosphatase activity.

The alkaline phosphatase content of tumor-bearing kidneys is quite unlike that of their normal controls. In the first place the neoplastic portions of all affected kidneys showed a negative alkaline phosphatase reaction. Secondly, areas adjacent to the neoplastic growth, although exhibiting the same general distribution of alkaline phosphatase as the normal controls, showed a marked reduction in enzymatic activity (Table I and Fig. 3).

It is noted in Table I, that the nontumorous portions of some tumor-bearing kidneys (36 and 39) showed a slightly greater phosphatase reaction than did the others. Upon examination we found that these tumors were simply solid masses of cells without lumina. This would indicate that the frog renal carcinoma does not exert its influence on the surrounding kidney tissue phosphatase in its earlier stages, *i.e.,* before the tumor has dif-

TABLE I.
Kidney Alkaline Phosphatase.

		Non-tumorous portions of kidney				
Frog expt.	Tumor	Proximal tubules	Collecting tubules	Glomerulus	Glomerular capsule	Distal tubules
			Normal			
1-14		++++	++++	+++	—	—
			Tumorous			
1-35 37-38, 40	—	+ to ++	+ to ++	+ to ++	—	—
36 and 39	—	+++	+++	++	—	—
Controls (technical)	—	—	—	—	—	—

The intensity of the phosphatase reaction was graded as follows: ++++ maximum, +++ large amount, ++ moderate, + slight, — negative.

ferentiated into the tubular form.

In all cases kidney sections at a distance from the neoplastic area showed a normal phosphatase content.

2. *Sites of acid phosphatase activity in normal and tumorous kidney.* Even when activated by 0.01 M manganous sulfate,[11] all portions of both the normal and neoplastic kidneys gave negative acid phosphatase reactions, except for an occasional positive glomerular reaction due to the phosphatase of the red blood corpuscles. These findings are comparable to those of other investigators[8,9] for normal kidneys.

Discussion. In all cases reported[7,12–14] the proximal tubules always show a strong positive reaction, whereas the distal convolutions are generally negative. The glomeruli are usually entirely negative. Only the glomeruli of the cat kidney[12] shows a positive alkaline phosphatase reaction similar to that which we observed in the frog glomeruli.

The high concentration of alkaline phosphatase in the proximal tubules suggests the possible role of this enzyme in sugar reabsorption since it has been shown that these tubules are the site of glucose reabsorption in the normal kidney.[15] Then too, it is well known that phlorhizin inhibits alkaline glycerophosphatase and also prevents the active reabsorption of glucose in the amphibian kidney.[16] A scheme for such a glucose-reabsorbing mechanism has been presented for certain mammalian kidneys.[14]

Our observation of a complete lack of alkaline phosphatase in the tumorous portions of the affected frog kidney, and its marked depletion in the adjacent renal tissue, indicates that the neoplasm interferes in some way with the metabolism of the surrounding tissue even though there is no microscopic evidence of its infiltration.

It must be emphasized that our results are based solely on the use of sodium-beta-glycerophosphate as the phosphoric ester hydrolyzed by the phosphatase of the kidney. The work of Dempsey and Deane[17] indicates that certain regions of the duodenum contain substrate-specific phosphatases, which suggests the possibility that other phosphatases might very well be demonstrable in the neoplastic amphibian kidney if other substrates and different pH ranges were employed.

Summary. It has been demonstrated for the first time, histochemically, that the normal kidney of the frog, *Rana pipiens*, possesses a strong alkaline glycerophosphatase reaction in its proximal tubules, especially at their brush borders, whereas the distal convolutions are negative. The glomeruli show a moderate alkaline reaction, categorizing the frog in a class with the cat (the only other animal thus far investigated that gives this reaction). With regards to the neoplastic kidney, the tumor cells themselves are alkaline phosphatase negative with an accompanying depletion of the enzyme in adjacent non-tumorous portions of the renal tissue.

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[11] Moog, F., *J. Cell. and Comp. Physiol.*, 1943, **32**, 95.

[12] Gomori, G., *J. Cell. and Comp. Physiol.*, 1941, **17**, 71.

[13] Bourne, G., *Quart. J. Exp. Physiol.*, 1943, **32**, 1.

[14] Wilmer, H. A., *Arch. Path.*, 1944, **37**, 227.

[15] White, H. L., and Schmitt, F. O., *Am. J. Physiol.*, 1926, **76**, 220.

[16] Walker, A. M., and Hudson, C. L., *Am. J. Physiol.*, 1937, **118**, 130.

[17] Dempsey, E. W., and Deane, H. W., *J. Cell. and Comp. Physiol.*, 1946, **27**, 159.

Both normal and tumorous kidneys are essentially negative for acid glycerophosphatase. The significance of these results is discussed.

Received August 28, 1949. P.S.E.B.M., 1949, **72**.

Depressing Effect of Inositol on Serum Cholesterol and Lipid Phosphorus in Diabetics.* (17437)

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The problem of the relation of serum cholesterol levels to human atherosclerosis is much debated. An agent capable of depressing serum cholesterol levels in hypercholesteremic individuals would offer a new approach to its study. There is some evidence to show that inositol is such an agent.

McHenry and Patterson[1] suggested that inositol has a specific effect on cholesterol metabolism; they pointed out that it is an effective lipotropic agent particularly when large amounts of cholesterol are present in the liver. Herrmann[2] found that inositol depressed blood levels of cholesterol and choles-

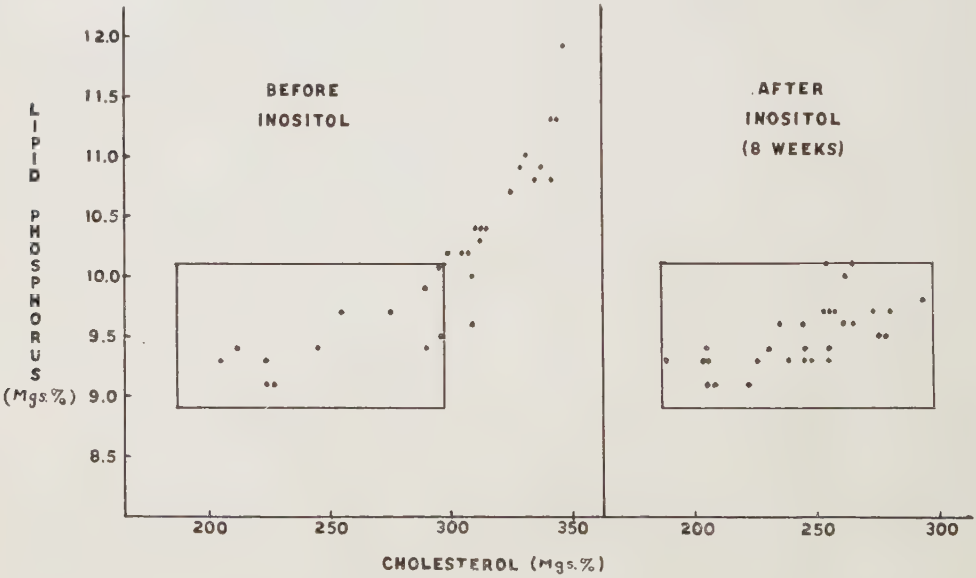

FIG. 1.

Values of serum total cholesterol and lipid phosphorus for 30 diabetic patients before and 8 weeks after inositol. Enclosed areas represent range of normal.

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* This study was supported in part by a grant from Commercial Solvents Corporation.

[1] McHenry, E. W., and Patterson, J. M., *Physiol. Rev.*, 1944, **24**, 129.

[2] Herrmann, G. R., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 436.

terol esters in old hens. Gephardt[3] found that in one case of xanthomatous biliary cirrhosis doses of 3 g of inositol per day for 3 months produced a marked drop in total lipids and some drop in cholesterol and cholesterol esters; the patient, however, received a low-fat diet concurrently. On the other hand, Shay[4] states that doses of 1.2 g a day for 3 weeks had no effect on blood cholesterol in human diabetics. Herrmann[5] found that 20 hypercholesteremic patients given 2 g of inositol a day for 25-30 days had an average drop of 19% and 17% of cholesterol and cholesterol esters respectively; one showed no change; he found also that there was a tendency for phospholipids to rise.

The action of inositol on serum lipids and on the atherosclerotic process is being studied in this hospital. The present paper reports its effect on certain serum lipids in a group of diabetic patients.†

Procedure. Total cholesterol and cholesterol esters[6] and lipid phosphorus[7] were determined on the blood serum of all experimental subjects. Similar determinations were made on 100 control subjects, consisting of staff physicians, nurses and hospital patients not known to have any disease affecting cholesterol metabolism.

Of the 30 diabetic patients used, 13 were selected because of high blood cholesterol levels previously established in this clinic; the remainder were picked at random from the diabetic clinic provided they fulfilled the criteria of fixed diet, fixed insulin dosage and adequate diabetic control for 6 months prior to beginning the experiment. Repeated de-

TABLE I.

The Mean, Range, Standard Deviation, and Standard Error of the Mean of Total Cholesterol, Cholesterol Esters and Lipid Phosphorus in Normals and in Diabetics Before and After Taking Inositol.

	Total cholesterol, mg %				Cholesterol esters, mg %				Lipid phosphorus, mg %		
	Mean	S.D.	S.E.	Range	Mean	S.D.	S.E.	Range	Mean	S.D.	S.E.
Normals (100)	236.3	±24.0	±2.4	(188-297)	167	±16.8	±1.7	(137-204)	9.29	±0.20	±0.02
Diabetics (30)											
Control	294	±43.5	±8.0	(205-346)	208	±29.6	±5.4	(144-246)	10.2	±0.71	±0.13
Inositol: 1 week	268	±33.9	±6.2	(198-321)	189	±24.0	±4.4	(138-225)	10.0	±0.61	±0.11
2 ,,	254	±31.3	±5.7	(188-303)	178	±22.6	±4.1	(136-213)	9.7	±0.42	±0.08
4 ,,	247	±27.7	±5.1	(186-292)	173	±19.3	±3.5	(129-207)	9.5	±0.31	±0.06
8 ,,	244	±26.8	±4.9	(189-293)	171	±18.7	±3.4	(131-208)	9.5	±0.27	±0.05

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[3] Gephardt, M. C., *Ann. Int. Med.*, 1947, **26**, 764.

[4] Shay, H., in discussion of Vorhaus, M. G., Gompertz, M. L., and Feder, A., *Am. J. Dig. Dis.*, 1943, **10**, 48.

[5] Herrmann, G. R., *Exp. Med. and Surg.*, 1947, **5**, 149.

† The authors are indebted to Dr. John H. Keating for his helpful advice and to Miss Stephanie Ilka for technical assistance in this investigation.

[6] Sperry, W., and Brand, F. C., *J. Biol. Chem.*, 1943, **150**, 315.

[7] Youngburg, G. E., and Youngburg, M. V., *J. Lab. and Clin. Med.*, 1930, **16**, 158.

terminations on these 2 diabetic groups were practically identical as far as mean, range and standard deviation of cholesterol fractions and lipid phosphorus were concerned; they are therefore considered as one unit.

At least 2 baseline total cholesterol, cholesterol esters and lipid phosphorus determinations were made on all diabetic patients before they were given inositol. They then began taking 3 g of inositol daily (two 0.5 g capsules after each meal); bloods were taken at intervals of 1, 2, 4, and 8 weeks thereafter.

Results. The range of normals is indicated by the enclosed area in Fig. 1. The mean, range and standard deviations are shown in Table I. No apparent variation was found with regard to age or sex and repeated determinations on the same subject showed little or no variation over a period of several months.

Before taking the inositol, the diabetic patients tended to show elevated serum total cholesterol, 18 (60%) being outside the range of normals as shown in Fig. 1, only 4 below the normal average, the rest high normal. Cholesterol esters were proportional to total cholesterol, the ratio of esters to total being relatively constant (68-73%). Lipid phosphorus tended to be high when the total cholesterol was high, but the correlation was not as consistent as with the cholesterol esters. No correlation between the level of cholesterol fractions or lipid phosphorus and the severity of diabetes or the accuracy of its control could be made.

In all of the diabetic patients fed the inositol a fall occurred in the serum cholesterol and lipid phosphorus. After 8 weeks (Fig. 1) all determinations were within the normal range. As shown in Table I, the total cholesterol tended to fall abruptly over the first 2 weeks, then to fall more slowly or become stationary. The higher initial total cholesterol showed the greater tendency to fall; thus, 17 diabetics with an initial total cholesterol above 300 mg% had an average drop of 69.2 mg% in 8 weeks, while the 13 diabetics below 300 mg% had an average drop of only 29.0 mg%. The proportion of esters to the total remained constant after inositol, and lipid phosphorus tended to fall in conjunction with fall in total cholesterol, contrary to Herrmann's findings. When the patients were taken off inositol, a gradual rise occurred in the determined serum lipids, but after 6 weeks none had returned to the baseline levels.

The only possible untoward effect of inositol observed occurred in one woman who complained of gastric distress; many of the patients reported a feeling of increased vigor and well-being. No consistent change in the diabetic status of any of the patients was noted.

Conclusions. Inositol is an effective agent in lowering serum cholesterol and lipid phosphorus in hypercholesteremic diabetics. Further study of its action and applications is warranted.

Received Sept. 14, 1949. P.S.E.B.M., 1949, **72**.

Metabolism of Some 9-Aminoacridine Derivatives. (17438)

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Many compounds in the 9-aminoacridine series have been synthesized for the purpose of studying their action against a variety of parasitic infections, and as disinfectants. The recent antimalarial survey[1] included a large number of these compounds. One of them was Acranil (SN-186), which was found to be only slightly active against avian malaria.[2] Previously[3-6] it had been used successfully for the treatment of human giardia infections and, more recently, against *Hymenolepis nana*.[7] Berberian has also found it quite effective for the treatment of human subjects infested with *Enterobius vermicularis*. Three closely related compounds which are superior to Acranil against oxyurids of mice,[8] and also as to acute toxicity for mice* are Win 2333, 2389, 2390. The structural formulae of these 4 compounds, to be discussed in this paper, are as follows:

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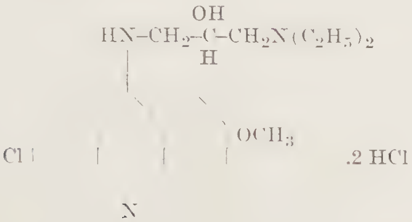
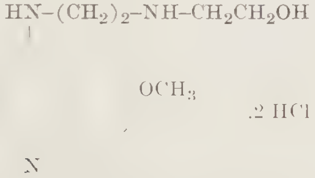
[1] Wiselogle, F. Y., A Survey of Antimalarial Drugs, J. W. Edwards, Ann Arbor, 1946, pp. 1321-1377.

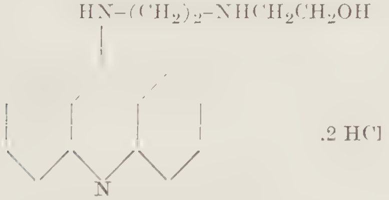
[2] Gingrich, W. D., and Fillmore, R. S., *Am. J. Hyg.*, 1942, **36**, 276.



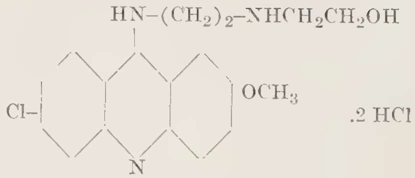
6-Chloro-2-methoxy-9-(2-hydroxy-3-diethylamino)-propylaminoacridine dihydrochloride
ACRANIL



9-(2-hydroxyethylaminoethylamino)-2-methoxyacridine dihydrochloride
WIN 2389



9-(2-hydroxyethylaminoethylamino)-acridine dihydrochloride
WIN 2333



6-Chloro-9-[3-(2-hydroxyethylamino)-propylamino]-2-methoxyacridine dihydrochloride
WIN 2390

Some information on the absorption, excretion, and tissue distribution of a typical 9-aminoacridine derivative is available as a result of the widespread interest in the use of Atabrine† for the therapy of human malaria.[9-12] It is known, for example, that the highest tissue concentrations for Atabrine, following oral administration to rats, are found in the liver, with lesser amounts in the spleen, lung, and kidney, in order of decreasing concentration.[9,10] In dogs, deposition in the tissues accounts for 10 to 30% of the oral dose.[10]

We have studied the 4 compounds listed above with respect to their absorption, excretion, and tissue-distribution, since these factors are important in determining their suitability for the treatment of susceptible parasitic infections.

Methods. The experimental subjects were male hooded rats from our colony, weighing 250-365 g each. They were kept in metabolism cages, one pair to each cage. In order to simulate as closely as possible the conditions used for the treatment of intestinal parasitic infections, they were allowed free access to our regular colony diet and water until the medication was given, but then only water was provided for the remainder of the experimental period. The drugs were given by

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[3] deMuro, P., *Acta Med. Scand.*, 1939, **102**, 17.

[4] Grott, J. W., *Schweiz. Med. Wochschr.*, 1939, **69**, 683.

[5] Friederich, L. V., *Gastroenterol.*, 1940, **65**, 24.

[6] Berberian, D. A., *Am. J. Trop. Med.*, 1945, **25**, 441.

[7] Berberian, D. A., *Am. J. Trop. Med.*, 1946, **26**, 339.

[8] Berberian, D. A., and Dennis, E. W., manuscript in preparation.

* Laboratory data, courtesy of Dr. J. O. Hoppe.

† Registered trade-marked name of Winthrop-Stearns, Inc., brand of quinacrine.

[9] Barlow, O. W., Auerbach, M. E., and Rivenburgh, H., *J. Lab. Clin. Med.*, 1945, **30**, 20.

[10] Army Malaria Research Unit, *Ann Trop. Med. and Parasitol.*, 1946, **40**, 173, 181, 368, 372, 472, 482.

[11] Dearborn, E. H., Oldham, F. K., Geiling, E. M. K., and Kelsey, F. E., *J. Pharm. and Exp. Therap.*, 1943, **78**, 120.

[12] Annegers, J. H., Snapp, F. E., Paskind, L., Ivy, A. C., and Atkinson, A. J., *War Med.*, 1943, **4**, 176.

stomach tube in 5 cc of water per kg body weight. This was enough water to dissolve the drug except for some doses of Win 2389 and 2390, which remained partly in suspension.

Twenty-four hours after medication samples of blood were drawn into oxalate by heart puncture. The animals were then sacrificed; the carcasses were frozen and stored until they could be analyzed. Urine and feces were collected for the 24-hour experimental period, the samples thus representing the combined excretions of one pair of rats. The blood and tissue samples, however, were usually analyzed for each animal. The methods used for the extraction and analysis have been described by Brodie *et al.*,[13,14] with the exception that in this work ethylene dichloride was used as a solvent in place of petroleum ether. As a routine measure the extracts were washed with 10% NaOH to remove metabolic products of the drugs, but no colored material was removed in this way. Acranil, Win 2389, and Win 2390 fluoresce in the visible range; they were determined in the Coleman photofluorometer using the filter system B-2, PC-9A. Win 2333 fluoresces only in the ultraviolet range. However, its behavior proved to be too erratic to use this property for its determination, therefore a colorimetric method was used.[‡] Since the sensitivity of the method was not as great as the fluorometric, it seemed inadvisable to attempt the determination of tissue concentrations of Win 2333 on any dose less than 200 mg/kg, and blood determinations were not made.

When the animals were dissected, the procedure was as follows: Aliquots of about 2 g of liver and lung were accurately weighed for extraction, also the whole spleen, and one whole kidney. If the stomach contents were sufficiently solid, a 2 g aliquot was taken, otherwise the entire contents were washed with water into the vessel to be used for the extraction. The intestines were washed out in a similar way, and to these washings were added the cecal contents. The analytical results are presented in Table I.

Discussion. It is evident that the number of experimental animals used was not sufficient to establish the tissue concentrations of each drug at each dose level with great precision. In about 2/3 of the paired analyses the agreement was very close; in the other third the variations were greater than one might wish (*i.e.*, 100% or more). In about half the aberrant figures the extent to which absorption had proceeded was clearly a factor (for example, Acranil, 500 mg/kg pair). In some other cases the extent of absorption alone could not account for the differences between paired animals (for example, Win 2389, 500 mg/kg pair). However, the purpose of the work was only to establish general trends, and with this in mind, the following generalizations can be made:

1. The tissue concentrations tended to increase with the dose level, as would be expected.

2. The urinary and fecal excretions did not show any consistent relationship to the dose level. Doubtless this is a reflection of the fact that considerable, but variable, amounts of the drugs remained in the gastrointestinal tract. However, it can be said that Acranil was excreted to the least extent in the urine, and Win 2389 to the greatest. Acranil was also excreted in the feces to the least, and Win 2389 to the greatest extent.

3. The blood concentrations of Acranil and Win 2390 increased with the dose level, and were of the same magnitude for equivalent doses. The blood concentrations of Win 2389 averaged as high, but were in a mixed order.

4. At any dose level chosen for comparison, Acranil accumulated in the tissues more than any of the other compounds, and Win 2333 accumulated the least; the affinity of liver for Acranil was particularly marked. In order of decreasing over-all tissue affinity, and of decreasing concentration in the individual tis-

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[13] Brodie, B. B., Udenfriend, S., and Baer, J. E., *J. Biol. Chem.*, 1947, **168**, 299.

[14] Brodie, B. B., Udenfriend, S., Dill, W., and Downing, G., *J. Biol. Chem.*, 1947, **168**, 311.

[‡] The extraction, washing, and transference into 0.1 N HCl were carried out in the specified way except that 10 cc of the HCl was used instead of 6 cc. The color of the HCl solution was then read in an Evelyn colorimeter using a 420 filter, comparing with standards containing 0.005-0.5 mg of Win 2333 in 10 cc of 0.1 N HCl.

TABLE I.

Absorption, Tissue Distribution, and Excretion of Some 9 aminoacridines 24 Hr after Oral Administration to Rats. Amounts in tissues,† gastrointestinal tract, excreta, and blood.

Compound, dose mg/kg	Compound, dose mg	Blood conc., mg/l	Stomach cont., mg	Intestinal and cecal cont., mg	Liver, mcg/g	Kidney, mcg/g	Spleen, mcg/g	Lung, mcg/g	% of dose in tissues and urine	% of dose in g. i. contents and feces
Acranil										
50	13.0	1.1	0.07	0.52	430	75	100	105	36.0	7.4
50	13.5	0.6	1.03	0.02	450	88	130	42	34.0	11.0
100	27.6	1.3	4.35	3.57	485	163	400	150	20.0	29.0
100	30.4	1.3	0.52	1.46	172	166	300	330	8.6	6.5
500	124.0	3.8*	24.3	10.0	2000	368	1040	535	2.8	15.0
500	139.0		86.0	9.9	880	378	565	310	8.4	69.0
WIN 2333										
200	66	—	3.1	11.2	53	17	54	12	2.2	27
200	64	—	17.6	1.9	33	15	40	11	1.8	36
500	182	—	80.6	4.9	47	49	41	11	2.0	50
500	140	—	18.0	1.4	98	33	54	30	2.9	17
WIN 2389										
50	13.8	1.6	0.02	4.60	23	7	2	2	3.3	74
50	14.0	1.4	0.07	4.62	18	10	16	4	3.3	74
100	29.7	0.8	1.72	6.55	17	30	43	14	6.4	39
100	27.6	0.7	0.01	1.53	17	32	86	32	7.0	17
500	135.0	3.2*	73.2	5.2	147	86	364	117	2.3	63
500	138.0		57.0	5.9	61	56	71	58	1.4	51
WIN 2390										
50	14.2	0.2	0.01	2.20	47	47	45	14	4.2	24
50	14.6	0.2	0.001	3.64	25	55	71	8	2.7	33
50	14.0	0.3	0.09	4.20	72	37	65	16	6.5	37
50	15.0	0.4	3.10	1.70	238	67	125	17	18.0	38
100	30.2	0.8	5.36	6.42	143	145	90	26	7.3	52
100	29.5	1.0	7.16	5.80	123	100	390	35	5.6	53
100	27.4	0.4	0.01	1.75	147	38	37	16	6.9	22
100	27.4	0.4	0.00	6.24	148	42	37	20	3.7	38
200	52.0	0.5	20.1	10.30	227	139	143	163	7.2	63
200	52.8	1.0	18.1	10.30	568	174	148	100	10.0	58
500	124.0	3.8*	17.6	75.4	256	124	233	100	2.7	76
500	133.0		51.6	26.0	279	81	70	80	2.7	60

* These animals were so close to a state of collapse that it was not possible to obtain sufficient blood for individual analyses.

† Tissue concentrations are based on wet weight.

sues the several compounds may be rated about as follows:

a. Acranil; liver > spleen > kidney > lung

b. Win 2390; liver > kidney > spleen > lung

c. Win 2389; spleen >liver > kidney > lung

d. Win 2333; liver > spleen > kidney > lung

It is of interest to compare the metabolism of these drugs with that of Atabrine, although the data available for comparison[9] were obtained with a higher dose than any used in this work: 675 mg/kg. None of these 4 drugs was absorbed as fast as Atabrine. Only at the lowest dose levels were as small (absolute) amounts found in the gastrointestinal tract as following Atabrine, and even less than 50 mg/kg of Win 2390 would have been required to produce an equivalent result. The reported tissue-distribution of Atabrine resembles that of Win 2389 in item[4] above. However, the latter compound was still only about half absorbed when the animals were killed. Win 2390 differed from Atabrine chiefly in giving higher liver concentrations. Even at the lowest dose level Acranil produced much higher liver concentrations than 675 mg/kg of Atabrine, and the other tissues contained amounts of the same order as following Atabrine.

It was noted that these compounds undergo some chemical alterations, probably in the liver, followed by excretion through the bile. This was shown by the fact that the ethylene dichloride extracts of both the intestinal (plus cecal) contents and of the feces contained an intensely yellow non-fluorescent substance which remained in the organic solvent, even when the latter was extracted with N HCl. This indicates that the basic side chain had been lost, leaving a neutral compound, possibly an acridone. A similar metabolic process has been observed by Hawking and Frazer in their studies of Miracil D.[15]

Summary. The absorption, excretion, and tissue-distribution of 4 compounds of the 9-aminoacridine series have been studied in rats at oral dose levels ranging from 50 to 500 mg/kg. At any dose level Acranil concentrates in the tissues to the greatest extent, while the other compounds follow in this order: Win 2390, 2389, 2333. For any single compound, the tissue concentrations ordinarily increase with the dose, the largest amount being found in the liver. In order of increasing rate of absorption the compounds are Win 2390, Win 2389, Acranil, Win 2333 but none of these is as completely absorbed within 24 hours as has previously been reported for Atabrine.

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[15] Hawking, F., and Ross, W. F., *Brit. J. Pharm. and Chemotherap.*, 1948, **3**, 167.

Received Sept. 16, 1949. P.S.E.B.M., 1949, **72**.

Regeneration of the Neural Portion of the Retina from Pigment Cells in Adult Urodele Eyes.* (17439)

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In previous experiments on grafted adult salamander eyes, it has been shown by the author,[1-3] that degeneration of the neural elements of the retina is followed by regeneration. The retina can be regenerated with sub-

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* Aided by grants from the James Hudson Brown Fund and the United States Public Health Service.

[1] Stone, L. S., and Zaur, I. S., *J. Exp. Zool.*, 1940, **85**, 243.

[2] Stone, L. S., and Farthing, T. E., *J. Exp. Zool.*, 1942, **91**, 265.

[3] Stone, L. S., and Ellison, F. S., *J. E. Zool.*, 1945, **100**, 217.

sequent return of vision at least 4 times in the same eye repeatedly transplanted.[2] The functional patterns associated with each of the retinal quadrants are reestablished and the visuomotor responses of the host are determined by the orientation of the graft.[4-7]

Regeneration of the retina in amphibians especially in larval urodeles has been reported previously by other investigators. As to its source of origin iris tissue has been claimed by some authors.[8-10] Retinal tissue at the ciliary margin[8,11] and ganglion cells in the fully differentiated retina[12] have been proposed as the source. One of the above mentioned investigators[8] believed that the regenerating retina in larval urodeles could also be derived from the pigmented epithelial layer.

An extensive study has been made to settle the question of the source of origin of the regenerating neural portion of the retina in the eye of the adult urodele, *Triturus v. viridescens*. In various groups of experiments over 500 grafts of iris tissue have been placed in the aqueous and vitreous chambers of the eye. None of them has given rise to retinal tissue. A detailed study was made of the degeneration and regeneration of the retina in 200 grafted eyes preserved at close intervals for at least 3 months after operation. Degeneration spreads throughout the neural retina during the first 3 weeks. In some cases destruction proceeds at different rates in various parts of the same eye and when degeneration overlaps early stages of regeneration it is more difficult to obtain a clear picture of the origin of the new retina. However, when degeneration is simultaneous in all parts it is clearly demonstrated that the new neural portion of the retina derives its origin from the surviving retinal pigment cells. This fact was demonstrated also in a study of 100 eyes from which the entire neural retina was successfully removed intact through a dorsal slit along the corneo-scleral junction after it had been detached from the underlying retinal pigment layer by a gentle stream of Ringer's solution emerging from a micropipette. The excised retinae were preserved and later served as controls for the eyes with regenerating retinae.

As soon as the pigment epithelial cells lose contact with neural retinal tissue they can be followed step by step as they change their structure and function. At first they become flattened oval densely pigmented bodies. Soon they increase in size and undergo mitosis. One daughter cell migrates inward, loses its pigment and gives rise to a chain of cells which later form the new neural retina. The other takes on the characteristics of a retinal pigment cell. Its pigment granules regain their ability to migrate in the presence of light only when the rod and cone cells of the new retina above them differentiate. It was also noticed in these experiments that the lost vitreous body was eventually replaced.

In another group of 63 eyes various injuries were made by inserting a micropipette through a wound in the cornea and removing by suction limited areas of the neural portion of the retina. In the larger wound areas the denuded pigment cells responded in the same manner as in those eyes from which all of the neural retina was eliminated. On the other hand if the wound area was small a rapid proliferation and migration of the heavily pigmented cells partially filled the space before depigmentation spread among them. As in other wound areas repair was carried to completion to replace the lost retinal tissue. There was always a sharp demarkation between the surrounding intact neural retina and the regenerating one. No evidence was found to support the idea that surviving cells of the neural portion of the retina bordering the wound ever contributed to the formation of the new retina.

To bring forth a response from the retinal

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[4] Stone, L. S., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 13.

[5] Stone, L. S., *Exp. Cell Research*, Sup. 1, 1949, 582, (Proc. 6th Int. Cong. Exp. Cytol.) 1947.

[6] Stone, L. S., *Ann. N. Y. Acad. Sci.*, 1948, **49**, 856.

[7] Sperry, R. W., *J. Comp. Neurol.*, 1943, **79**, 33.

[8] Wachs, H., *Roux Arch.*, 1920, **46**, 328.

[9] Sato, T., *Roux Arch.*, 1933, **130**, 19.

[10] Monroy, A., *Roux Arch.*, 1939, **139**, 536.

[11] Zolokav, M., *Rev. Suisse d. Zool.*, 1944, **51**, 443.

[12] Bücklers, M., *Arch. f. Opthol.*, 1933, **130**, 257.

pigment cells it is not essential to destroy or completely remove the neural portion of the retina. If a segment of the fully differentiated or regenerating neural retina becomes detached as an elevated fold it may survive. In the arch beneath it the retinal pigment cells immediately give rise to a secondary neural retina.

In 59 experiments segments of the eye wall containing the retinal pigment layer were implanted in the vitreous chamber of recipient eyes from which the lens had been removed. They also showed the development of neural retina from the pigment layer. It is therefore concluded that the regenerating neural portion of the retina in the adult urodele eye has its only source of origin from the cells of the retinal pigment layer.

Received Sept. 22, 1949. P.S.E.B.M., 1949, **72**.

Enhancement of Hemolytic Activity of Complement by Polyethylene Glycols.* (17440)

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In the course of an investigation designed to test the ability of various substances to prevent the deterioration of complement, it was found that certain of the polyethylene glycols greatly enhance the hemolytic activity of complement. The polyethylene glycols, a series of polymers with the general formula $HOCH_2(CH_2OCH_2)_xCH_2OH$, are soluble in water and in many of the aromatic hydrocarbons. The compounds with average molecular weight up to 700 are liquids; those with average molecular weight above 1000 are wax-like solids, sold under the trade name "Carbowax".† This paper constitutes a preliminary report on the effect of the polyethylene glycol carbowax-4000 on the hemolytic activity of complement, as determined by complement titrations in the presence and absence of this compound.

Procedure. Complement titrations were carried out according to the 50% hemolysis end-point method developed by Mayer *et al.*,[1] and Kent *et al.*[2] The reaction mixtures, 20 ml in volume, had the following composition: 1) 1 part complement, the dilution so adjusted as to yield final dilution values ranging from 1:3300 to 1:950; 2) 1 part standardized suspension of sensitized erythrocytes; 3) either 2 parts of buffered saline,[3] or 2 parts of an 8% solution of carbowax-4000 in buffered saline. The sensitized erythrocytes were well mixed with either buffered saline or carbowax-4000 solution, and then the complement added dropwise with constant agitation. All components were kept in ice-water until time of incubation. The reaction mixtures thus prepared were incubated at 37°C for 60 minutes, with frequent agitation. After centrifugation of the mixtures, readings of per cent hemolysis in the supernates were made at 5500 Å in a Coleman spectrophotometer, an optical density of .500 representing complete hemolysis. Titrations were carried out in duplicate. Controls were set up of 1) sensitized erythrocytes and carbowax-4000, 2) unsensitized erythrocytes and carbowax-4000, and 3) complement, unsensitized erythrocytes and carbowax-4000.

Results and discussion. None of the control tests showed any hemolysis. The results of the titrations are shown in Fig. 1, in which

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* This work was supported by a grant from the National Tuberculosis Association.

† Carbon and Carbide Chemicals Corporation, New York.

[1] Mayer, M. M., Eaton, B. B., and Heidelberger, M., *J. Immunol.*, 1946, **53**, 31.

[2] Kent, J. F., Bukantz, S. C., and Rein, C. R., *J. Immunol.*, 1946, **53**, 37.

[3] Mayer, M. M., Osler, A. G., Bier, O. G., and Heidelberger, M., *J. Exp. Med.*, 1946, **84**, 535.

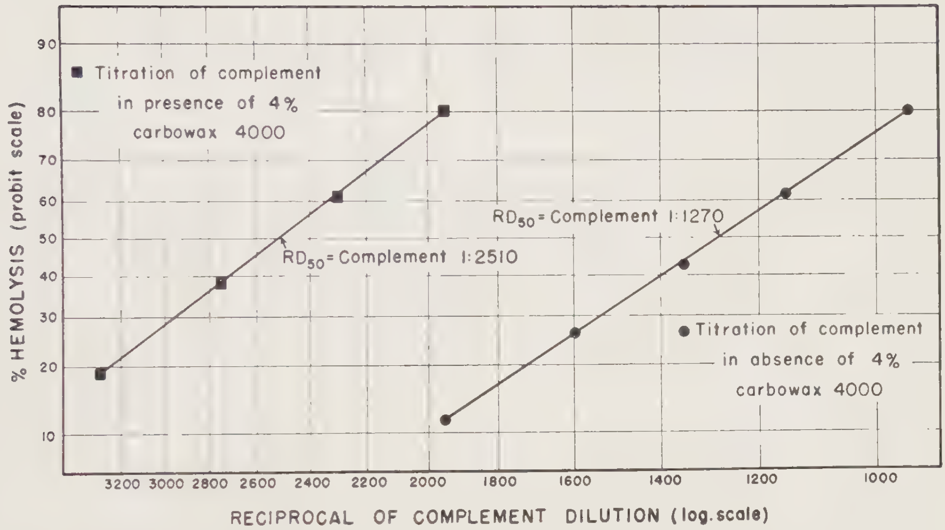

FIG. 1.

Titration of complement in the presence and absence of 4% carbowax-4000. The complement dilutions referred to on the abscissa represent final dilutions, not the dilutions added to the reaction mixture.

the probit of the percent hemolysis is expressed as a function of the log of the dilution of complement, in the presence and absence of 4% carbowax-4000. The dilutions of complement, with or without 4% carbowax-4000, calculated to yield a required per cent hemolysis, as determined from the curves of Fig. 1, are set forth in Table I. A striking difference in the ability of a given dilution of complement to lyse sensitized erythrocytes in the presence or absence of carbowax-4000 is evident. Within the range of values for per cent hemolysis which can be used with assurance in this technic, the two curves overlap only at a complement dilution of 1:1950. At this point, complement alone gives 11.5% hemolysis; complement and 4% carbowax-4000 together, give 79.9% hemolysis, an increase of nearly 700%. The RD_{50} unit (that amount of complement required to lyse 50% of the sensitized erythrocytes) in the absence and presence of 4% carbowax-4000 is 1:1270 and 1:2510 respectively. The fact that only approximately half the usual amount of complement is necessary for 50% hemolysis, with 4% carbowax-4000 present, may be of practical importance for complement-fixation tests, a possibility which is being investigated. The other polyethylene glycols are being tested for similar activity, and a detailed study of the general reaction described in this paper is under way. At present no explanation of the mechanism of the enhanced hemolytic activity of complement in the presence of polyethylene glycols can be offered, though it is perhaps worthwhile to speculate on a possible relation between the fat-solvent nature of these compounds and the lipoidal components of the erythrocytic membrane.

TABLE I.

Comparison of Reciprocals of Dilutions of Complement Calculated to Give a Required Per Cent Hemolysis in Presence and Absence of 4% Carbowax-4000.

	Reciprocal of dilution of complement	
% hemolysis	Without carbowax	With carbowax
10	2000	3750
20	1710	3260
30	1525	2960
40	1390	2720
50	1270	2510
60	1165	2330
70	1065	2140
80	950	1950

The author wishes to acknowledge the technical assistance of Miss Dolores Smith.

Received Sept. 26, 1949. P.S.E.B.M., 1949, **72**.

Effect of Methionine Deficiency upon Enzyme Activity in the Rat.* (17441)

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The effects of low protein intake upon enzyme activity in the rat have been studied recently by several workers. Seifter *et al.*[1] have shown that during complete dietary protein deprivation the loss of D-amino acid oxidase and arginase activity in rat liver occurs faster than loss of general liver protein. Westerfeld and Richert[2] have reported that xanthine oxidase activity is decreased in rats fed low protein diets. We[3] have observed that liver xanthine oxidase activity is very sensitive to subtle changes in nitrogen intake. Adult rats fed a diet completely adequate for maintenance may show a liver xanthine oxidase content far below normal. Moreover it was shown that addition of 0.25% DL-methionine to the diet increased xanthine oxidase activity to normal.

The present paper is a preliminary report concerning the effects of a true amino acid deficiency upon liver enzyme activity. Because of the observed strongly limiting effects of methionine upon liver xanthine oxidase activity, this amino acid was chosen for the first study. Subsequently we expect to report the effect of other amino acid deficiencies upon liver enzyme activity.

Experimental. Eighteen male, weanling albino rats of the Holtzmann strain were placed on a good stock ration for 10 days. They were then given, for one week, a purified ration reported by Ramasarma *et al.*[4] to be the best for growth of young rats. Briefly, this ration consisted of a purified amino acid mix (16%), salts, the known vitamins, corn oil, and sucrose. The animals were fed and watered *ad libitum* during the 7-day period. At the end of that time the animals were divided into 2 groups of 9 animals each. One group was given the complete ration, and the other group was fed the complete ration less methionine. In order to prevent poor food intake due to the methionine-deficiency both groups of animals during the experimental period were force fed in 3 daily portions an amount of the ration equal to the average intake of the animals during the *ad libitum* feeding period. The rations were taken up in distilled water to make a thick suspension and were fed by stomach tube.

After 14 days of the force-feeding period a methionine deficiency was distinctly evident in the animals receiving the methionine deficient diet as evidenced by bleeding of the feet and mouth and general loss of weight. During this period 3 animals of the group receiving the methionine-deficient diet died. The animals receiving the complete diet gained weight throughout the period and showed no detrimental signs because of the regimen.

When the enzyme experiments were begun it was thought best to sacrifice the animals as quickly as possible in order to prevent variations due to different lengths of time on the diets. For this reason 3 animals from the methionine-deficient group were sacrificed on one day; the remaining 3, the next. The animals receiving the complete ration were sacrificed in the following order on consecutive days: 4 animals, 3 animals and 2 animals.

The animals were killed by decapitation and the livers immediately removed. A portion of each liver was quickly weighed out on a delicate torsion balance. The portions of

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* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation; by funds from the Robert Gould Research Foundation, Cincinnati, Ohio, and by a grant from the Nutrition Foundation, Inc., New York, N. Y.

[1] Seifter, S., Harkness, D. M., Rubin, L., and Muntwyler, E., *J. Biol. Chem.*, 1948, **176**, 1371.

[2] Westerfeld, W. W., and Richert, D. A., *Fed. Proc.*, 1949, **8**, 265.

[3] Williams, J. N., Jr., and Elvehjem, C. A., *J. Biol. Chem.*, in press.

[4] Ramasarma, G. B., Henderson, L. M., and Elvehjem, C. A., *J. Nutrition*, 1949, **38**, 177.

TABLE I.
Effect of Methionine Deficiency Upon Enzyme Activity in Rat Liver.

Enzyme	Ration	No. of animals	Activity based on wet liver	Activity based on liver N
Xanthine oxidase	Basal	3	0*	0*
		3	0	0
	Basal + methionine	4	141	4150
		3	134	4100
		2	137	4120
Succinic dehydrogenase	Basal	3	14.8†	500†
		3	12.5	540
	Basal + methionine	4	18.0	550
		3	18.6	560
		2	18.6	580
Endogenous respiration	Basal	3	1.06†	35.0†
		3	1.07	45.0
	Basal + methionine	4	1.51	44.5
		3	1.48	45.6
		2	1.56	48.5

* Units = μ 10$_2$ per hr per g wet liver.
† Units = μ 10$_2$ per hr per mg wet liver.

liver from the animals sacrificed at one time were pooled in 5 volumes of ice-cold 0.039 M sodium potassium phosphate buffer, homogenized for 3 minutes, and strained through gauze. This homogenate was used in the xanthine oxidase determinations according to the method of Axelrod and Elvehjem.[5] A portion of the homogenate was diluted with buffer to give a 10% homogenate, which was employed for measuring succinic dehydrogenase according to the method of Schneider and Potter.[6] Both enzymes were assayed using a Warburg bath maintained at 30°C. Aliquots of each homogenate were taken for nitrogen determinations in triplicate using a microKjeldahl procedure.

Results. The enzyme values as presented in Table I are based both upon liver wet weight and liver nitrogen. Since enzymes are protein in nature, loss or gain of enzyme activity as compared to general liver protein can be obtained by basing enzyme activity upon liver nitrogen. This also negates the effects of dilution of liver by glycogen or lipid. Endogenous respiration of the liver homogenates was calculated from the first 10-minute period of oxidation before xanthine was added to the flasks for measuring xanthine oxidase activity. Endogenous respiration is reported since it is believed to be a measure of general liver metabolic rate.

As shown in the table a methionine deficiency induces a significant decrease in all 3 of the activities measured when activity is based upon wet weight of the liver. It was observed that the livers of the methionine deficient animals were somewhat infiltrated with fat, which probably accounts for the more noticeable decrease of wet weight enzyme activity in the amino acid deficient rats. The comparative loss of enzyme activity with general liver protein is not so pronounced in the case of succinic dehydrogenase activity. It appears that activity of this enzyme, which according to Schneider[7] is centered mainly in the mitochondrial elements of the liver cell, is held almost as tenaciously as other liver protein.

The striking disappearance of xanthine oxidase activity during a methionine deficiency is difficult to explain. Seifter *et al.*[1] have reported that during protein starvation the riboflavin content of the liver is lowered. This would not, however, explain such a marked loss in flavin enzyme activity. It appears that the extreme lability of xanthine oxidase activity is due to several unknown factors. It is difficult to believe that activity of this enzyme is lost because of its lack of necessity to the animal since purine metabolism is believed to be very important in animal functions.

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[5] Axelrod, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, 1941, **140**, 725.

[6] Umbreit, *et al.*, Manometric technics and related methods for the study of tissue metabolism, 1945.

[7] Schneider, W. C., *J. Biol. Chem.*, 1946, **165**, 585.

As shown in the table, general endogenous respiration of the livers of the animals receiving the complete ration and those receiving the methionine deficient ration are essentially the same. Stare and Elvehjem[8] had reported that endogenous respiration of chick and rat tissue is practically unaffected by various vitamin deficiencies. It has been recently reported[9] that endogenous respiration of chick liver is unaffected by folic acid or vitamin B_{12} deficiencies. It thus appears that while dietary conditions have little effect upon the total endogenous metabolism of the liver individual enzyme activities may be varied significantly by the diet.

Summary. It has been observed that a methionine deficiency in the rat reduces liver succinic dehydrogenase activity slightly, completely reduces liver xanthine oxidase activity, and has practically no effect upon endogenous respiration of liver tissue *in vitro*.

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[8] Stare, F. J., and Elvehjem, C. A., *Am. J. Physiol.*, 1933, **105**, 655.

[9] Williams, J. N., Jr., Nichol, C. A., and Elvehjem, C. A., *J. Biol. Chem.*, 1949, **180**, 689.

Received Sept. 26, 1949. P.S.E.B.M., 1949, **72**.

Lipotropic Effects of Vitamin B_{12} Concentrate. (17442)

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It has recently been reported that crude liver extract exerts a lipotropic effect in rats with dietary induced liver injury.[1] The effect of the liver extract was not due to stimulation of the appetite with a resultant increase in protein intake, nor did the lipotropic effect seem to be related to the choline content of the liver extract. It was also demonstrated that other supplements. with a higher choline content than that of liver extract, exert a lesser degree of lipotropic activity when compared with liver extract.[2] In the present studies the lipotropic effect of a vitamin B_{12} concentrate was determined in rats fed a high-fat diet.

Methods. Male rats of the Sprague-Dawley strain, weighing between 115 and 149 grams were used. The high-fat diet (51% lard) and the control diet (6% lard) as recently described,[3] were fed *ad libitum* for the period listed in Tables I and II. When the animals were placed on the synthetic diets injections of vitamin B_{12} concentrate were begun and were administered subcutaneously 3 times a week.* At the end of the experiment sections were taken from the left lobe of the liver and stained with hematoxylin and eosin. Frozen sections were also made and stained with Sudan III. The remainder of the liver was analyzed for total fat.[4]

Results. In the first study treatment with a vitamin B_{12} concentrate, in doses of 0.2 μg of vitamin B_{12} 3 times a week, reduced the average fat content of the liver from 31.5%

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[1] Hall, C. A., and Drill, V. A., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 3.

[2] Drill, V. A., and Hall, C. A., *Am. J. Med. Sci.*, in press.

[3] Hall, C. A., and Drill, V. A., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 202.

* The Oleum Percomorphum used in the diet was kindly supplied by Mead Johnson and Company.

The vitamin B_{12} concentrate was supplied by Dr. T. H. Jukes of Lederle Laboratories Division of the American Cyanamid Company.

[4] Outhouse, E. L., and Forbes, J. C., *J. Lab. and Clin. Med.*, 1939, **25**, 1157.

TABLE I.
Effect of Vitamin B_{12} Concentrate on Hepatic Changes of Rats Fed High-Fat Diet for 29 Days.

No. of rats	Avg wt change, g	Avg liver wt, g	Avg liver wt g/100 g rat	No. animals with hepatic fatty changes* 0	1+-2+	3+-4+	Avg liver fat, %
High fat diet, untreated							
6	55 ± 6.12	17.1 ± 1.25	8.1 ± 0.47	0	0	6	31.5 ± 1.18
High fat diet, + B_{12} conc. ≎ 0.2 µg B_{12} 3 times a wk							
5	48 ± 8.13	10.4 ± 0.66	5.1 ± 0.17	1	4	0	16.3 ± 1.47

* Grading of fatty change.
0 = None or only a rare large droplet.
1+ = Few large droplets in each lobule.
2+ = More than 1+, but less than half of lobule involved.
3+ = Over half of lobule involved, but some cells fat-free.
4+ = Almost no fat-free cells.

TABLE II.
Effect of Vitamin B_{12} Concentrate on Hepatic Changes of Rats Fed High-Fat Diet for 66 Days

No. of rats	Avg wt change, g	Avg liver wt, g	Avg liver wt g/100 g rat	No. animals with hepatic fatty changes* 0	1+-2+	3+-4+	Avg liver fat, %
Normal diet, control							
4	160 ± 3.14	12.1 ± 1.13	4.0 ± 0.37	3	1	0	5.6 ± 1.08
High fat diet, untreated							
8	43 ± 9.67	15.7 ± 1.07	8.5 ± 0.62	0	1	7	24.6 ± 0.59
High fat diet, + B_{12} conc. ≎ 1.0 µg B_{12} 3 times a wk							
10	84.6 ± 8.44	10.7 ± 0.71	4.7 ± 0.22	8	1	1	7.8 ± 0.89

* Fatty changes graded as in Table I.

in untreated animals to 16.3% in treated rats (Table I). Histological examination also showed a partial effect of the treatment. The vitamin B_{12} concentrate also prevented the increase in liver weight that usually occurs in untreated rats fed a high-fat diet. There was no significant difference in the gain in weight between the two groups of animals.

Treatment with a higher amount of vitamin B_{12} concentrate (1 µg of vitamin B_{12} 3 times a week) over a period of 66 days demonstrated a marked lipotropic activity of the concentrate. The fat content of the liver of the treated animals was not significantly different from the amount present in rats fed a normal diet (Table II). Histological examinations of the liver also demonstrated the lipotropic activity of the vitamin B_{12} concentrates. The weight of the liver was similarly affected by the treatment and was in the range of the normal controls. Furthermore, there was a significantly greater weight gain in the treated group as compared with the animals fed a high-fat diet alone.

Discussion. A vitamin B_{12} concentrate administered over a period of 29 and 66 days to rats receiving a high-fat diet showed a significant lipotropic effect. This effect is comparable to the previous results obtained with liver extract.[1,2] Both vitamin B_{12} concentrate and liver extract contained a small amount of choline and methionine. An injection of vitamin B_{12} concentrate, containing 1 gamma of vitamin B_{12}, supplied only 0.5 mg of choline, 0.19 mg of methionine, and 0.06 µg of folic acid. Based on 3 injections of the concentrate per week, each rat received only 0.21 mg of choline and 0.08 mg of methionine per day. These amounts of choline and methionine *per se* are ineffective in preventing the dietary induced fatty livers. The rats injected with 0.2 µg of vitamin B_{12} received only one-fifth of the amount of these added factors. The nature of the lipotropic action of the vitamin B_{12} concentrate is not known. It is possible that the effect of vitamin B_{12} and choline are interrelated, particularly as it has been reported that dietary choline has a specific sparing action on vitamin B_{12} as measured by the growth of the chick.[5] Further studies on the possible lipotropic action of crystalline vitamin B_{12} are in progress.

Conclusion. A concentrate of vitamin B_{12} exerted a marked lipotropic effect when injected into rats receiving a high-fat diet. This lipotropic effect was not due to the small amount of choline present in the concentrate.

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[5] Schaefer, A. E., Salmon, W. D., and Strength, D. R., PROC. SOC. EXP. BIOL. AND MED., 1949, **71**, 202.

Received Sept. 26, 1949. P.S.E.B.M., 1949, **72**.

A Micro Version of the Barcroft-Warburg Tissue Metabolism Apparatus.* (17443)

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Often it is desirable to measure oxygen consumption or other gaseous exchange of amounts of tissue too small to yield adequate changes in the usual 15-17 ml Warburg vessel. Several different types of apparatus have been constructed for this purpose: Warburg mentioned one of 4 ml capacity,[1] the Fenn type[2] is well known, and in recent years more diversified models have appeared (cf. evaluation by Tobias[3]).

A miniature form of the standard Warburg type, shown in Fig. 1, was designed of 2.5 to 3.3 ml capacity which has proved so convenient that this brief report is being made to note its availability.† The actual size is about 20 mm bottom diameter and 35 mm height, with the main vessel orifice being standard taper 14/20 and the side-bulb opening 6/15. A lip is placed on the center well, facing the side-bulb, for ease in pouring if the barium hydroxide-hydrochloric acid procedure is used for measurement of CO_2 production in the same vessels in which O_2 consumption is determined. Four hooks, instead of the conventional two, are routinely placed on all of our flasks and manometers in order to avoid the need for repairs when one hook is accidentally knocked off.

The manometer has a graduated portion 150 mm long, made from tubing about 1 mm inner diameter, to increase the total capacity as little as possible. Calibration is carried out by the use of mercury, although it is desirable to

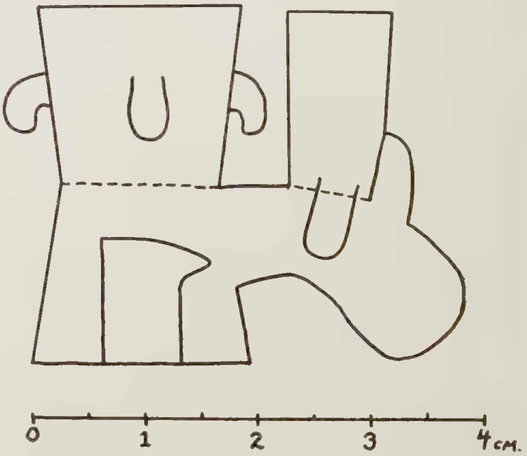

FIG. 1.
Diagram of micro respiration vessel.

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* This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships, National Institutes of Health, U. S. Public Health Service.

[1] Warburg, O., *Biochem. Z.*, 1923, **142**, 317.

[2] Fenn, W. O., *Am. J. Physiol.*, 1928, **80**, 327.

[3] Tobias, J. M., *Physiol. Rev.*, 1943, **23**, 51.

† The author wishes to thank the E. Machlett Company, 220 East 23rd St., New York City, for its cooperation in designing and fabricating these vessels.

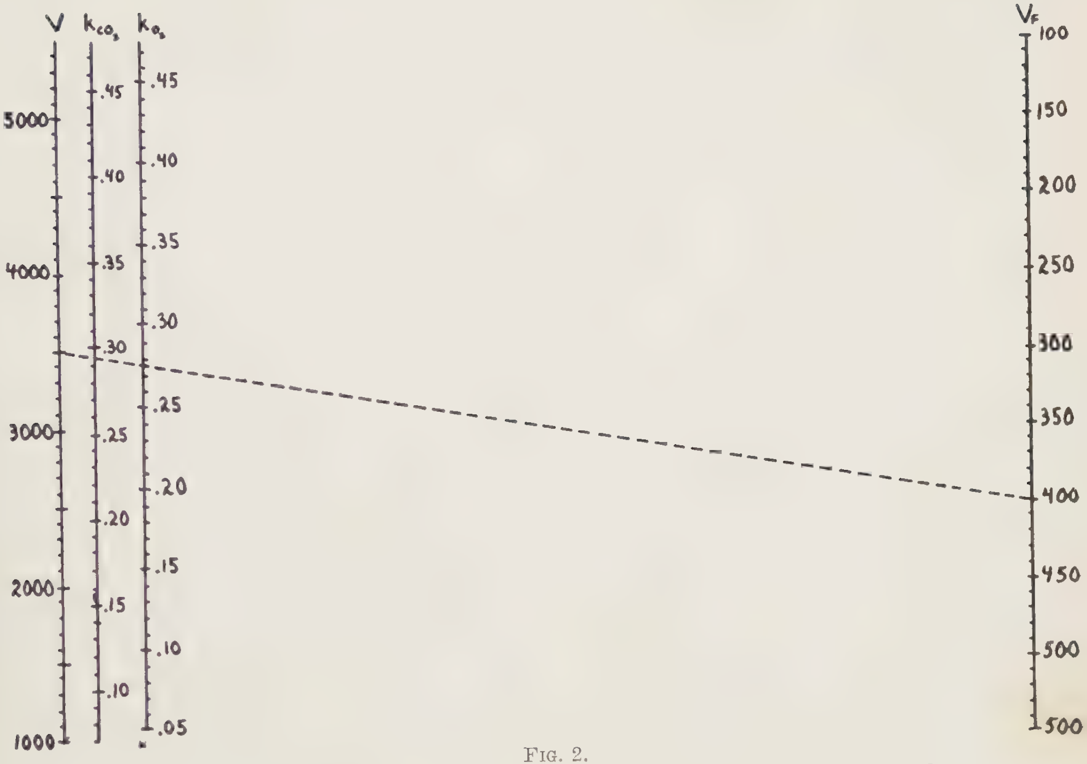

FIG. 2.
Nomogram for determining k_{O_2} and k_{CO_2} values for micro respiration vessels.

fill the vessel and upper part of the manometer down to the 75 mark to be used as zero with one filling of mercury. This technic avoids the second stage of filling from a scratch mark to the zero with its possible errors.

In operation, the movement of fluid in the open limb of the manometer is quite rapid because of the small gas volume in the vessel for expansion or contraction. Tipping over substrate from the side-bulb requires a scrupulously clean glass surface, lest a grease layer accentuate the difficulty of pouring from such a small cup. Volumes of solution which have been found convenient to use are 300 μl of buffered medium, 50 μl of alkali (usually 10% KOH) in the center well and 50 μl of substrate solution in the side-bulb. For complete CO_2 and O_2 determinations, 75 μl of $Ba(OH)_2$ may be placed in the side-arm and 100 μl of HCl in the center well. These small volumes of fluid may be introduced into the desired parts of the vessel by means of a tuberculin syringe and needle, Kirk micro-pipette and delivering device, or glass Mohr pipette with drawn-out tip.

A nomogram constructed according to Dickens[4] is presented in Fig. 2 to facilitate obtaining vessel constants at 37°C for vessels from 1 to 5.5 ml in volume. It should be emphasized that V is the total capacity of vessel and manometer to the midway mark used as the zero for the constant volume readings. In the example shown, the total volume of vessel and manometer limb is 3500 μl and the fluid volume in the vessel is 400 μl. The k_{O_2} for this experiment is 0.277 and the k_{CO_2} is 0.294. Thus the change in manometer reading due to gaseous exchange would be approximately 3.5 times as great as the actual volume changes.

Table I lists Q_{O_2} comparisons for several rat tissues obtained in this laboratory between these vessels and the conventional 17 ml size. The metabolic activities correspond closely. The sensitivity difference is about the 4- to

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[4] Dickens, F., *Biochem. J.*, 1945, **39**, 427.

TABLE I.
Comparison of Amounts of Rat Tissue and Q_{O_2} Values in Standard and in Micro Vessels.

Tissue	Micro vessel		Macro vessel	
	Tissue wt, mg*	Q_{O_2}†	Tissue wt, mg*	Q_{O_2}†
Diaphragm	40	0.98	150	1.02
Heart	40	0.76		
Skeletal muscle	60	0.57	300	0.45
Kidney	15	3.32	80	3.25
Liver	40	1.29	200	1.34
Thyroid	20	0.95		

* Wet weights are used throughout.
† cu mm O_2/mg wet wt/hr.

5-fold one would anticipate from the difference in constants. There is little reason for using the micro-vessels for a tissue as metabolically active as kidney, because of the great care required in weighing the approximately 15 mg samples. The real value is in such cases as skeletal muscle, with a low rate of metabolism, or thyroid gland, with so little total tissue available.

Summary. A micro modification of the standard Warburg vessel for the study of gaseous exchange of tissues has been described. As would be anticipated from the decrease in size from 17 ml to about 3 ml, the amounts of tissue required for satisfactory results can be reduced to about one-fifth those usually used.

An advantage over other more sensitive apparatus is that the manometer supports are interchangeable with the standard Barcroft-Warburg supports, so the same water-bath and shaking device can be used for both.

Received Sept. 27, 1949. P.S.E.B.M., 1949, **72**.

Administration of Folic Acid to Patients Without Anemia.* (17444)

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In view of reports[1-11] showing the appearance of neurologic disease following folic

TABLE I.
Distribution of Cases Treated for More Than One Year. ♂ 24 ♀ 21.

Age	No.
10-19	1
20-29	3
30-39	8
40-49	8
50-59	8
60-69	9
70-79	7
80-89	1

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* The folic acid used in this study was furnished by the Lederle Laboratories Division, American Cyanamid Company.

[1] Davidson, L. S. P., and Girdwood, R. H., *Lancet*, 1948, **254**, 360.

[2] Hall, B. E., and Watkins, C. H., *J. Lab. and Clin. Med.*, 1947, **32**, 622.

[3] Heinle, R. W., and Welch, A. D., *J.A.M.A.*, 1947, **133**, 739.

[4] Jacobson, S. D., Berman, L., Axelrod, A. R., and vander Heide, E. C., *J.A.M.A.*, 1948, **137**, 825.

[5] Meyer, L. M., *Bull. N. Y. Acad. Med.*, 1946, **22**, 484.

[6] Meyer, L. M., *Blood*, 1947, **2**, 50.

[7] Meyer, L. M., *Am. J. Clin. Path.*, 1948, **18**, 811.

[8] Ross, J. F., Belding, H., and Paegel, B. L., *Blood*, 1948, **3**, 68.

[9] Vilter, C. F., Vilter, R. W., and Spies, T. D., *Proc. Central Soc. Clin. Research*, 1946, **19**, 26.

[10] Vilter, C. F., Vilter, R. W., and Spies, T. D., *J. Lab. and Clin. Med.*, 1947, **32**, 262.

TABLE II.
Hematologic Data on Cases Treated for More than One Year.

	Avg before treatment	Avg after treatment	Increased cases	Decreased cases
Hb.	12.1 g	12.2 g	24	21
R.B.C.	4,540,000	4,480,000	17	28
W.B.C.	7,100	6,900		

acid administration to patients having macrocytic anemia, a long-term study in nonpernicious anemia persons was instituted.

Methods. Seventy individuals were studied for periods of from 5 to 13 months each, for a total of 768 patient months. Forty-five of the 70 were followed for more than one year. The cases were selected from hospital and outpatient population. Patients suffering from the following diseases were included, such as: rheumatoid arthritis, hypertension, rectal polyp, epidydimitis, alcohol neuropathy, lues, tracheitis, neurasthenia, bronchiectasis, cholecystitis, bronchial asthma, osteoarthritis, Beck's sarcoid, cretinism and arteriosclerosis. The criteria used in selection were availability for follow-up and ability to cooperate. To reduce variables, no patients with pernicious anemia or diabetes mellitus were accepted. No attempt was made to alter general disease, as regards diet and medication. Table I shows age and sex distribution of the 45 cases studied for more than one year. Each person was given 20 mg of folic acid daily, orally, in the form of one tablet. During the period of study some patients improved from their primary disease and were discharged. This accounts for the variability in period of survey. At monthly intervals all cases were checked for hematologic data and neurologic status, which included a complete blood count and hemoglobin, gait, tendon reflexes, touch, vibration, and temperature sense, Romberg sign and muscle grip.

Results. There were no changes noted during the period of study in the group of 70 patients No patient developed any subjective or objective sign of combined sclerosis or peripheral neuritis. Hematologic data are given for the sake of completeness. Table II shows averages of hemoglobin, red cell count and white cell count for the 45 patients studied over one year. No interpretation can be offered since diet, medication and primary disease variables must be as significant as folic acid administration.

Comment. It is known from several studies[2-6,8-11] that folic acid does not protect the pernicious anemia patient from combined sclerosis. Other reports[1,7] have shown the appearance of combined sclerosis symptoms in patients with macrocytic anemia of gastrointestinal origin on folic acid therapy. No reports have demonstrated appearance of neurologic disease in non-macrocytic anemia patients taking folic acid. This long-term study, constituting 768 patient-months on 20 mg daily dosage of folic acid showed no neurologic toxicity. Most patients taking the drug reported a feeling of well-being. The blood counts in these non-macrocytic anemia patients were not significantly changed.

Summary. A study of long-term folic acid administration in 70 non-macrocytic anemia patients was made. Signs related to sub-acute combined sclerosis or peripheral neuritis did not develop in any of the group.

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[11] Wagley, P. F., *New England J. of Med.*, 1948, **238**, 11.

Received Sept. 28, 1949. P.S.E.B.M., 1949, **72**.

Simplified Technic for Preparing Blood Group Specific Substance A. (17445)

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Since the blood group specific substances are carbohydrate like in nature it seemed logical that such a simple technic as preparing a protein free filtrate by the Folin-Wu method would be an easy way to prepare blood group substance A from Difco neopeptone. As will be shown the blood group substance was not destroyed by the reagents used in the Folin-Wu adaptation. The product which is obtained compares favorably in potency and purity with preparations made with more difficult procedures.

Materials and Methods. The starting material for this Folin-Wu adaptation was a 5% solution of Difco neopeptone in distilled water. For every 30 ml of neopeptone solution 15 ml of 10% sodium tungstate was added, followed by the slow addition of 15 ml of 2/3 N H_2SO_4. After all the sulfuric acid was added the container was shaken and then left to stand for 10-15 minutes. The filtrate which contains the blood group specific substance was dialyzed against cool running water until a negative test for tungstate, sodium and sulfate was obtained. The various chemical tests such as the picric acid, Molisch, etc. were run on this dialysate (recorded under results). Two procedures were used to prepare the final product: either, (1) the dialysate was precipitated with 10 volumes of acetone, centrifuged and dried in a vacuum desiccator, or 2) dialyzed against several changes of physiological saline and stored in the liquid form. The former product was tested in inhibition studies on a dry weight basis and compared with products prepared by the method of Goebel.[1] It should be recalled that this investigator prepared blood group specific substance A from neopeptone by multiple precipitations with 95% alcohol, using the deproteinizing method of Sevag.[2] In addition to comparing the latter product of our method with the product obtained by Goebel's method, it was compared with the blood group substance A content of a commercial product (Sharp-Dohme).

Anti-sera. Anti A sera for tests was prepared so as to contain as nearly as possible 8 units of isoagglutinin per unit volume.[3]

Inhibition tests. One drop of anti-A sera was added to one drop of the particular strength of the blood group specific substance. The tube was shaken and left to stand for 45 minutes at room temperature. One drop of a 2% suspension of A cells was added, the tube was shaken and left to stand for one hour. The presence or absence of agglutination was determined macroscopically. The technic is a modification of Wiener's[3] method for testing for secretors.

Results. The saline dialysate compared favorably with the commercial preparation in the inhibition tests for blood group specific substance A. The dialysate of our preparation inhibited agglutination through a titer of 256, while the commercial product inhibited agglutination through the titer of 128.

On a dry weight basis our product inhibited the agglutinating properties of 1 drop of anti-A sera when only 1 mg was present in 256 ml of physiological saline. The Goebel product which we prepared by his method gave inhibition when only 1 mg was present in 205 ml of physiological saline.

The Goebel method in our hands as well as the Folin-Wu technic gave a product free of amino acids according to the picric acid, Millons, Biuret and Hopkins-Cole tests. The commercial product gave a strongly positive Millons test. The Molisch reaction was strongly positive with all products.

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[1] Goebel, W. F., *J. Exp. Med.*, 1938, **68**, 221.

[2] Sevag, M. C., *Biochem. Z.*, 1934, **273**, 419.

[3] Wiener, A. S., 1945, Blood Groups and Transfusions, Charles C. Thomas, Springfield, Ill.

Discussion. The use of tungstate and sulfuric acid to prepare a protein free filtrate, while not removing the carbohydrate is a standard procedure in blood analysis work.[4] The fact that the blood group specific substances are carbohydrate like lends them admirably to this procedure. The method was probably never attempted before because of the nature of the reagents used since there was some doubt in our mind initially as to whether the blood group specific substance would withstand this treatment. It is not claimed that a superior product is obtained by the Folin-Wu technic in comparison to the method of Goebel, but the procedural simplicity of the former makes it a method of choice which should be easily used when animal tissues are the source of blood group specific substances.

Summary. A method for preparing blood group specific substance A from Difco neopeptone by the Folin-Wu technic is described and comparisons with other preparations are made. The method is simple and the product obtained compares favorably in purity and potency with products obtained by other methods.

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[4] Andrews, J. C., and Kyker, G. C., 1947, A Laboratory Manual of Biological Chemistry, Edwards Brothers, Inc., Ann Arbor, Mich.

Received October 4, 1949. P.S.E.B.M., 1949, **72**.

Influence of Adrenal Cortical Steroids and Related Compounds on Sodium Metabolism.* (17446)

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In a previous paper from this laboratory[1] it was demonstrated that as little as one microgram of desoxycorticosterone produced a significant retention of sodium in the adrenalectomized male rat. This communication is concerned with the study of various adrenal cortical steroids and related compound on sodium metabolism by the same technic. These steroids include 11-dehydrocorticosterone, 17-hydroxycorticosterone, corticosterone, pregnanetrione-3, 11, 20, allopregnanetriol-3 (β), 17 (α), 21-one-20, alloprenanepentol-3 (β), 11(β), 17(α), 20, 21-3(β), 20, 21 triacetate, Δ^4-prenenol-21-trione-3,12,20 acetate, testosterone, estradiol, and pregnanetrione-3,12,20.

Animals, Materials, Methods. The rats were obtained from Carworth Farms. They were bilaterally adrenalectomized in one stage under ether anaesthesia. The animals subsisted exclusively on a chow diet both before and after adrenalectomy. When more than 24 hours elapsed between adrenalectomy and the day the experiment was run the rats were given normal saline in place of ordinary drinking water until the morning of the experiment.

The test material was injected subcutaneously in 0.25 cc of corn oil One hour later the rats received subcutaneously, 2 cc of a solution containing sodium chloride and the radiosodium.† The dose of sodium chloride was 35 μg per gram of body weight. The animals were placed in glass collecting cages and the urine collected for 6 hours. The urine was dried and the concentration of radiosodium in the residue determined as described previously.[1]

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* Supported in part by research grants from the Division of Research Grants and Fellowships of the Public Health Service, and Sharp and Dohme, Inc., Glenolden, Pa.

[1] Dorfman, R. I., Potts, A. M., and Feil, M. L., *Endocrinology*, 1947, **41**, 464.

† The radiosodium was supplied by the Monsanto Chemical Company, through the U. S. Atomic Energy Commission.

TABLE I.
Influence of 11-Dehydrocorticosterone Acetate on Excretion of Na in the Adrenalectomized Male Rat.

Control			Experimental			
Mean B.W. g ± S.E. (No.)	Mean Na excretion % ± S.E.	Amt, μg	Mean B.W. g ± S.E. (No.)	Mean Na excretion % ± S.E.	Change, %	t
115 ± 5 (9)	4.57 ± 1.0	25	113 ± 3 (9)	5.04 ± 0.75	+10	
115 ± 5 (9)	4.57 ± 1.0	75	119 ± 3 (9)	3.39 ± 0.45	—26	1.03
115 ± 5 (9)	4.57 ± 1.0	100	122 ± 3 (9)	3.79 ± 0.54	—17	
124 ± 3 (8)	4.30 ± 0.55	100	137 ± 4 (9)	4.88 ± 0.42	+14	0.81

TABLE II.
Influence of 17-Hydroxycorticosterone on Excretion of Na in Adrenalectomized Male Rats.

Control			Experimental			
Mean B.W. g ± S.E. (No.)	Mean Na excretion % ± S.E.	Amt, μg	Mean B.W. g ± S.E. (No.)	Mean Na excretion % ± S.E.	Change, %	t
135 ± 3 (9)	4.59 ± 0.48	25	142 ± 5 (9)	4.88 ± 0.93	+ 6	
128 ± 4 (8)	1.48 ± 0.27	50	124 ± 3 (8)	2.44 ± 0.26	+65	2.570
127 ± 5 (9)	1.51 ± 0.25	50	120 ± 4 (9)	2.67 ± 0.52	+77	2.020
127 ± 5 (9)	1.51 ± 0.25	200	121 ± 6 (9)	2.18 ± 0.25	+44	1.88
139 ± 6 (8)	6.69 ± 0.55	200	143 ± 5 (8)	7.06 ± 1.06	+ 6	

The results were expressed as the amount of radiosodium excreted compared to the total amount of radiosodium administered in per cent. The effect was measured by comparing the mean percentage excretion of the experimental animals to the mean percentage excretion of the control animals which were run simultaneously.

Following is a list of compounds studied and their source.

Ciba Pharmaceutical Products, Inc.; Desoxycorticosterone, Desoxycorticosterone acetate, Testosterone, Estradiol.

Dr. R. D. H. Heard, McGill University; Δ^4-pregnenol-21-trione-3, 12, 20-21-acetate

The Upjohn Company; Allopregnane-3(β), 17(α) 21-one-20, 17-Hydroxycorticosterone, Corticosterone

Dr. E. C. Kendall, Mayo Clinic; 11-Dehydrocorticosterone acetate, Corticosterone, 17-Hydroxycorticosterone

Dr. T. F. Gallagher, Memorial Hospital; Pregnanetrione-3,11,20

Dr. T. Reichstein, University of Basle; Allopregnanepentrol-3(β), 11(β), 17(α), 20, 21, 3(β), 20, 21-triacetate

Dr. D. Prins, Cleveland Clinic; Pregnanetrione-3,12,20

Results. In a previous publication (Dorfman, Feil, and Potts[1]) it was shown that desoxycorticosterone produces a significant sodium retention in adrenalectomized male rats in amounts as low as one microgram. The desoxycorticosterone acetate produced retention at a concentration of 25 μg but lower concentrations of the ester have not been studied.

Table I lists 4 experiments on the influence of 11-dehydrocorticosterone acetate on sodium metabolism in the adrenalectomized male rats. No significant changes in sodium excretion were found at concentrations of 25 to 100 μg

TABLE III.
Influence of Δ^4-Pregnenol-21-Trione-3,12,20-21-Acetate on Excretion of Na in Adrenalectomized Rats.

Control			Experimental			
Mean B.W. g ± S.E. (No.)	Mean Na excretion % ± S.E.	Amt, μg	Mean B.W. g ± S.E. (No.)	Mean Na excretion % ± S.E.	Change, %	t
132 ± 4 (9)	1.53 ± 0.23	100	127 ± 3 (9)	1.75 ± 0.17	+14	0.76
124 ± 3 (8)	4.30 ± 0.55	400	126 ± 5 (5)	4.21 ± 0.50	— 2	
169 ± 3 (11)	3.52 ± 0.41	1340	177 ± 4 (12)	2.36 ± 0.39	—33	2.03

TABLE IV.
Activities of Various Steroids on Na Excretion in Adrenalectomized Rats.

Compound	Dosage, μg	Type of activity
Desoxycorticosterone	1*	Retention
Desoxycorticosterone acetate	25*	"
Δ^4-Pregnenol-21-trione-3,12,20-21-acetate	100	Negative
	400	"
	1340	Retention
17-Hydroxycorticosterone	25	Negative
	50	Increased excretion
	200	Negative (?)
11-Dehydrocorticosterone acetate	100	Negative
Pregnanetrione-3,11,20	200	"
Corticosterone	400	"
Allopregnanepentol-3(β), 11(β), 17(α), 20,21-3(β),20,21-triacetate	800	"
Allopregnanetriol-3(β), 17(α), 21-one-20	800	"
Testosterone	2000	"
Estradiol	2000	"
Pregnanetrione-3,12,20	2000	"

* Lowest concentration studied.

of this adrenal cortical steroid. The influence of 17-hydroxycorticosterone on the excretion of sodium in the adrenalectomized rat is represented in Table II. At a level of 25 μg a mean excretion of 4.88% ± 0.93% was found as compared to the control value of 4.59% ± 0.48% which represented an insignificant change of +6%. When the dose of 17-hydroxycorticosterone was increased to 50 μg an increase in sodium excretion of + 65 and + 77% respectively in 2 experiments was observed. The t values for these experiments were 2.570 and 2.020. At 200 μg a tendency toward increased excretion of sodium was observed in one experiment (+44%, t = 1.888) while in a second experiment no significant change was observed.

Δ^4-pregnenol-21-trione-3,12,20 was studied at dose levels of 100, 400, and 1340 μg (Table III). At a dose level of 1340 μg a statistical significant retention of 33% (t = 2.03) was found. This is the first instance of biological activity being found for this compound. At 100 and 400 μg dose levels no significant change in sodium excretion was observed.

All other steroids studied were inactive at the doses tested. These included: pregnanetrione-3,11,20 (200 μg), corticosterone (400 μg), allopregnanepentol-3(β), 17(α), 11(α), 20, 21-3(β), 20,21-triacetate (800 μg), allopregnanetrione-3,12,20 (2000 μg), testosterone (2000 μg), and estradiol (2000 μg).

Discussion. A compilation of the results is presented in Table IV. Desoxycorticosterone is by far the most active substance tested having given positive results at one microgram. The acetate produced significant retention at 25 μg. No lower concentrations have been tested. Δ^4-pregnenol-21-trione-3,12,20 acetate has been found to cause significant sodium retention at 1340 μg and a negative effect at 400 μg. Thus the introduction of the 12 keto group results in a compound possessing about 2 to 5% of desoxycorticosterone. 17-Hydroxycorticosterone produced an increased sodium excretion at 50 μg but the effect was either minimized or obliterated at 200 μg. Increased urinary excretion of sodium has been observed previously for 17-hydroxycorticosterone and 17-hydroxy-11-dehydrocorticosterone in normal dogs and rats[2] and in partially depancreatized rats.[3] Ingle and coworkers[4] have found that relatively large doses of ether 17-hydroxycorticosterone or 17-hydroxy-11-dehydrocorticosterone caused an immediate increase in sodium excretion during the first 24 hours but that the sodium excretion returned to the control levels in spite of continued treatment.

Summary. Eleven steroid compounds have been studied as to their influence on sodium excretion in adrenalectomized rats. Desoxycorticosterone caused significant retention at one microgram, and the acetate was effective at 25 micrograms, the lowest concentration studied. Δ^4-pregnenol-21-trione-3,12,20-21-acetate gave a significant retention at 1340 micrograms. 17-Hydroxycorticosterone caused a significant excretion of sodium.

The following steroids were found to be negative: 11-dehydrocorticosterone acetate (100 μg), pregnanetrione-3,11,20 (200 μg), corticosterone (400 μg), allopregnanepentol-3 (β), 11(β), 17(α), 20, 21-3(β), 20, 21-triacetate (800 μg), allopregnanetriol-3(β), 17 (α), 21-one-20 (800 μg), testosterone (2000 μg), estradiol (2000 μg), and pregnanetrione-3,12,20 (2000 μg).

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[2] Thorn, G. W., Engel, L. L., and Lewis, R. A., *Science*, 1941, **94**, 348.

[3] Ingle, D. J., and Thorn, G. W., *Am. J. Physiol.*, 1941, **132**, 670.

[4] Ingle, D. J., Sheppard, R., Evans, J. S., and Kuizenga, M. H., *Endocrinology*, 1945, **37**, 341.

Received October 13, 1949. P.S.E.B.M., 1949, **72**.

Evaluation of Autolyzed Mouse Brain Tissue Method for Isolation and Adaptation of Poliomyelitis Virus. (17447)

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A method for isolation and adaptation of poliomyelitis virus by direct passage into mice was described by Milzer and Byrd.[1] The method consists in mixing suspensions of infected feces or central nervous system (CNS) material with an equal amount of a 10% suspension of autolyzed normal mouse brain tissue (ABT) and injecting the mixture intracerebrally into white mice. It is obvious that a usable method for primary isolation of poliomyelitis virus without the use of expensive monkeys would present a great advantage. The authors undertook, therefore, to verify this method and carried out a total of 19 experiments with mice and 4 with cotton rats.

Materials and Methods. Feces. Five specimens were taken from poliomyelitis patients, but not tested for virus content. The presence of poliomyelitis virus in 7 other stool specimens was demonstrated by monkey inoculation prior to adaptation experiments. (The stool specimens were obtained through courtesy of Dr. Franklin H. Top, Herman Kiefer Hospital, Detroit, and Dr. Joseph L. Melnick, Yale University).

Monkey adapted strains. Spinal cords of monkeys infected with poliomyelitis virus strains Buffalo, Mahoney and Tennessee were obtained through courtesy of Dr. Thomas Francis, Jr., University of Michigan. The virus content was confirmed by monkey inoculation prior to adaptation experiments.

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[1] Milzer, A., and Byrd, C. L., *Science*, 1947, **105**, 70.

Lansing strain. In view of the claim[1,2] that the use of the autolyzed brain tissue technic results in a shortening of the incubation period in mice inoculated with the Lansing strain virus, this strain was also included in some of the experiments.

Mice and Cotton Rats. White mice bred at the Michigan Department of Health (Rockland Swiss mice) were used in 9 experiments, and the CFW (Carworth Farms Webster) strain of mice in 10 experiments. Eastern cotton rats bred at the Michigan Department of Health were used.

Preparation of the Autolyzed Normal Mouse Brain Tissue Suspensions. The technic described by Milzer and Byrd was used. The autolysis was achieved by keeping the sacrificed mice at room temperature for 16-17 hours prior to the removal of the brains. In most cases, the temperatures were between 22°C and 25°C. However, in some experiments, temperatures lower or higher than those indicated were recorded.

In view of the high rate of bacterial contaminations, the mouse brains were emulsified in pairs and the sterility of each pool tested separately by aerobic and anaerobic culture (blood agar plates and thioglycolate broth). The suspensions were kept in the refrigerator for not more than 24 hours, and the sterile suspensions pooled. They were filtered through several thicknesses of gauze before use.

Diluents. In most of the experiments, either nutrient broth of the Baltimore Biological Laboratory (BBL, pH 6.83 - 7.25), or Tyrode's solution (pH 7.3 - 7.9), or both, were used as diluents. In 2 experiments, Bacto nutrient broth (pH 6.8 - 6.85), and in 2 other experiments thioglycolate sterility broth (pH 7.2 - 7.35), were used.

Organization of the experiments. Groups of 10 to 20 mice were inoculated with: (1) stool suspension plus ABT in nutrient broth, (2) stool suspension plus ABT in Tyrode's solution, (3) stool suspension plus nutrient broth (no ABT), (4) stool suspension plus Tyrode's solution (no ABT), (5) ABT plus nutrient broth (no stool), (6) ABT plus Tyrode's solution (no stool). The same setup was used for infected monkey cords and the Lansing strain (mouse cords), and for other diluents. Two or, more frequently, 3 specimens were used in each experiment. The controls mentioned under (3) and (4) were included for each specimen, while those under (5) and (6) served as general controls. The same specimens were used for the experiments repeatedly up to 10 times. In the first 5 experiments, 2 groups of mice were used for each preparation, one inoculated i.c., the other i.c. and i.p. In all the other experiments, only the i.c. route was used.

The experiments with cotton rats were set up in the same way. However, smaller groups of animals (3 to 5) were used. The observation period for both mice and cotton rats was 40 days.

Single and repeated injections, blind passages. There were experiments with a single inoculation, and others with 2, 3, or 4 inoculations (suggestion of Dr. Milzer). In 2 experiments, there was 1 inoculation of virus-ABT mixture, followed by a series of 5 blind passages (no ABT was used for the blind passages).

Experimental. Experiments with mice. The Lansing strain was tested 10 times in Rockland strain mice and once in CFW mice. Nutrient broth (Bacto and BBL) and Tyrode's solution were used as diluents. In no case was any shortening of the incubation period observed.

In 12 experiments in which the stools with previously demonstrated virus content as well as the 3 monkey adapted strains were tested repeatedly, none of the mice developed any symptoms. In 2 of these experiments, the 3 monkey adapted strains were concentrated 7 times by ultracentrifugation. The final dilution of the concentrated virus (after addition of ABT) was 35%. BBL nutrient broth, Tyrode's solution and thioglycolate broth were used as diluents in these experiments.

In 4 experiments some of the mice inocu-

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[2] Milzer, A., Byrd, C. L., and Levinson, S. D., Abstracts of Papers Presented at the 47th General Meeting of the Soc. Am. Bacteriologists, 1947, 74.

lated with mixtures of ABT and either stool suspensions or suspensions of the strains Mahoney and Tennessee, developed partial paralysis. Other symptoms such as ruffled fur, humped back, and tremors were present in some cases and absent in others. In all these cases, the character of paralysis was quite different from that observed in mice inoculated with the Lansing strain. Bacteriological examinations of the CNS revealed bacterial infection in all these cases. Histopathological examinations revealed fibrinopurulent exudate covering brain and medulla, or diffuse infiltration of the meninges, or multiple cortical abscesses, but no lesions characteristic for poliomyelitis.*

In one of the experiments, a control mouse inoculated with a suspension of ABT in nutrient broth (no poliomyelitis virus containing material) developed paralysis similar to that observed with the Lansing strain or with some strains of mouse encephalomyelitis virus. There was no bacterial contamination. Pathological examinations did not show any poliomyelitic lesions. The CNS tissue of this mouse was passed into other mice which, however, did not develop any symptoms.

In 2 other experiments, 2 different virus strains producing paralysis in mice were isolated. One of the strains (referred to as ABC) was isolated from the CNS of one of the control mice inoculated i.c. with a suspension of ABT in nutrient broth. The mouse developed paralysis on the 14th day after inoculation. On further mouse passages the shortest incubation period was 2 to 3 days. Several of the mice inoculated i.p. developed paralysis. The shortest incubation period by the i.p. route was 6 days. Since no material was used which could possibly contain human poliomyelitis virus, no monkey was inoculated with this virus.

Another neurotropic virus (referred to as N-C strain) was isolated under the following circumstances. A stool suspension (received through courtesy of Dr. Melnick) was mixed with a suspension of ABT in Tyrode's solution and inoculated i.c. into mice. None of the mice developed symptoms. A few mice were sacrificed at random and serial blind passages were carried out with thioglycollate broth as diluent and without addition of ABT. At the 4th blind passage one mouse developed paralysis after an incubation period of 19 days. On repeated passages the incubation period became shorter (10 days or more). There was no difference whether or not ABT was added to the inoculum. None of the mice inoculated with this strain by the i.p. route developed symptoms. Since poliomyelitis virus containing stool material was used for the very first inoculation of mice, a Rhesus monkey was inoculated with the N-C strain. A 10% suspension of virus was used, and the monkey received 1.5 ml i.c., a total of 4 ml intranasally and a total of 16 ml i.p. The monkey did not develop paralysis, tremors, or any other symptoms. There was no rise of temperature at any time.

Experiments with cotton rats. All 4 experiments with cotton rats were entirely negative. Three monkey adapted strains and several of the stools with previously demonstrated virus content were used for these experiments.

Discussion. From the data presented in this report, based on experiments carried out with a total of over 5,000 mice and 340 cotton rats, it is clear that the authors were not able to confirm the effectiveness of the autolyzed normal mouse brain tissue method either in the primary isolation of poliomyelitis virus by direct passage into rodents, or in the adaptation of monkey adapted strains to mice or cotton rats, or in the shortening of the incubation period in mice inoculated with the Lansing strain.

In a series of 19 experiments with mice and 4 with cotton rats, 2 neurotropic viruses were isolated. It seems clear, however, that the ABC strain is of mouse origin, since no poliomyelitis virus containing material was injected in this case. The circumstances of isolation of the N-C strain were different and do not exclude the possibility of it being a strain of poliomyelitis virus. The subsequent test in a monkey, however, inoculated with a large dose of virus, makes this highly improbable.

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* The histopathological examinations were carried out by H. E. Cope, M.D., Clinical Pathologist, Division of Laboratories, Michigan Department of Health.

Summary and conclusions. The effectiveness of the autolyzed normal mouse brain method for isolation and adaptation of poliomyelitis virus was investigated in 19 experiments with mice and 4 experiments with cotton rats. Three monkey adapted strains (Tennessee, Mahoney and Buffalo) and 12 stool specimens from poliomyelitis patients were used. The Lansing strain was also included in the experiments.

In the experiments with mice, 2 strains of neurotropic viruses were isolated. However, the evidence presented suggests that the strains isolated were of mouse origin. The cotton rat experiments were entirely negative. A shortening of the incubation period in mice inoculated with the Lansing strain was not observed.

The data presented do not confirm the effectiveness of this method for the isolation or adaptation of poliomyelitis virus.

Received October 4, 1949. P.S.E.B.M., 1949, **72**.

Actions of 2,6-Diaminopurine in Mice, Rats, and Dogs.* (17448)

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Recent studies have shown 4-aminopteroylglutamic acid (4-amino-PGA) to cause changes in hematopoietic tissues and intestinal mucosa which closely resemble lesions found in folic acid-deficient manmals.[1,2] These findings and the fact that 4-amino-PGA and its congeners act as competitive antagonists of folic acid (PGA) in certain microorganisms[3-6] have led to the conception that the agents damage tissues in mammals by interference with metabolic functions of PGA.[1,2,5,6]

Studies of derivatives of naturally occurring purines and pyrimidines have uncovered another antagonist of PGA in microorganisms,[7] namely, 2,6-diaminopurine, which also appears active in higher organisms. Thus 2,6-diaminopurine delays development of an experimental leukemia in mice[8] and inhibits estrogen-induced growth in chick oviduct.[9] The purine is related structurally to 4-amino-PGA insofar as both compounds are condensed ring-systems containing 2,4-diamino-pyrimidine. These observations suggested that the purine might act as an antagonist of PGA in mammals. The following studies were undertaken to determine in detail, sites of action of 2,6-diaminopurine in mice, rats and dogs and to compare the lesions obtained with those seen after administration of 4-amino-PGA.

Procedure. Mice, rats, and dogs of the same strains and corresponding in sex, weight, age and maintenance to animals used in previous studies with 4-amino-PGA[1,2] were sub-

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* The authors wish to acknowledge support of this study by the National Institute of Health, U. S. Public Health Service.

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TABLE I.
Toxicity of 2,6-Diaminopurine Following Intraperitoneal Administration.

Species	No. of inj.	Dose, mg/kg/day	Mortality	Day of death 1	2	3	4	5–14	Approx. LD50† mg/kg/day
Mouse	1	395	12/12	10	2				170
		198	11/12		9	2			
		99	0/12						
Mouse	5*	158	12/12		5	3	4		90
		79	2/12			2			
		40	0/12						
Rat	1	316	12/12	12					100
		158	11/12	7	2	2			
		79	3/12	1		1		1	
		40	0/12						
Rat	5*	158	12/12	6	6				45
		79	12/12		7	4	1		
		40	2/12					2	

* Administered on 5 successive days.
† Estimated on log-probability paper.

jects of the present investigation. Procedures followed herein were also similar to those described previously. The purine‡ was used as the lactate or hydrochloride which is more soluble than the free base and was injected at physiologically tolerable temperatures after heating to dissolve the salts.

Course of intoxication in mice and rats. Animals receiving large intraperitoneal doses of 2,6-diaminopurine ($\geqq$ 500 mg/kg) evidenced ataxia, weakness and dyspnea within 5 to 15 minutes. The initial signs of intoxication increased progressively in severity and animals succumbed with respiratory failure within 1 to 6 hours. Single lethal doses, less than 500 mg/kg caused death usually within 24 to 48 hours (Table I). Such animals lost approximately 20% of initial weight and exhibited severe diarrhea prior to death. Rats and mice which survived single, large doses lost less than 10% of initial weight during the first 24 to 48 hours and were fully recovered before the end of the first week. Repeated doses of diaminopurine had similar, though delayed, effects (Table I). On the basis of toxicological data the response of mice and rats to diaminopurine may be differentiated from that following 4-amino-PGA. Regardless of the dose employed, animals receiving 4-amino-PGA did not respond with early manifestations of intoxication and survived for at least 3 days after poisoning. Furthermore, repeated administration of the PGA-analog proved cumulative in effect and more toxic than injection of single doses.[1] Table I suggests that repeated doses of diaminopurine equivalent to large fractions of the acute LD_{50} were tolerated without fatality.

Lesions in rats. Animals receiving 100 mg/kg/day, and sacrificed after 24 and 48 hours showed at autopsy distended gastrointestinal canals filled with yellow fluid. Femoral bone marrow contained purple fluid instead of a gelatinous, greyish red substance. Findings in peripheral blood and smears of femoral marrows obtained at time of sacrifice are found in Table II. The findings in peripheral blood indicated no significant change in leucocytes or reticulocytes. Hemoglobin concentrations were elevated indicating hemoconcentration. Sections of sternal and femoral marrow showed progressive depletion of total nucleated elements estimated to be about 50% after 24 hours and 80% after 48 hours. Studies of

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‡ The authors are grateful to Dr. George H. Hitchings and his associates of the Wellcome Research Laboratories for ample supplies of 2,6-diaminopurine, and also to Dr. George B. Brown and coworkers of the Division of Protein Chemistry, Sloan-Kettering Institute, for provision of samples of the agent used in the early part of these studies.

TABLE II.

Hematological Data from Rats Treated with 2,6-Diaminopurine. (No. of animals used shown in parentheses).

					Peripheral blood				Femoral marrow				
Group	Dosage, mg/kg/day	No. of inj.	Day of sacrifice		GRCYT, 10^3/mm^3	LYCYT, 10^3/mm^3	HB, g/100 cc	RETIC, %	N-GRCYT, %	EOS-GRCYT, %	MYEL, %	ERYTH, %	LYCYT, %
Control*				Mean	1.2(11)	5.8(11)	13.4(11)	11(6)	33(12)	6(12)	19(12)	35(12)	9(12)
				range	0.4-2.7	1.9-12.4	12.1-15.5	7-17	10-57	2-20	10-30	16-70	3-21
Acute	100	1	1		2.4	0.4	17.6	8.0	21	3	12	16	48
					3.2	1.7	18.0	15.3	21	3	12	20	44
					4.6	3.0	16.5	6.3	14	2	8	26	50
					3.4	2.6	17.6	7.2	37	3	5	16	39
		2	2		1.0	1.2	—	3.0	7	6	15	40	32
					0.1	2.9	15.8	3.2	17	1	19	28	35
					0.6	2.1	13.8	8.2	6	3	9	33	49
I	20,40	12-17	17-25	Mean (8)	3.0	8.7	13.2	1.3	29	15	24	23	9
				range	0.6-4.7	2.8-19.8	7.0-15.4	0-4.9	8-54	3-26	14-40	7-35	2-30
II	20,40,79	15-21	22-30	Mean (8)	1.4	1.9	13.7	0	10	8	23	17	42
				range	0.4-2.8	0.4-4.4	10.7-18.1	0	2-25	3-14	6-31	3-35	26-65

* From data published previously.[1]

EOS-GRCYT, eosinophilic granulocytes.

GRCYT, granulocytes.

LYCYT, lymphocytes.

MYEL, metamyelocytes, myelocytes, and myeloblasts.

ERYTH, nucleated erythroid cells.

HB, hemoglobin.

N-GRCYT, neutrophilic granulocytes.

RETIC, reticulocytes.

sections and differential counts of nucleated elements in smears of femoral contents (Table II) revealed increased proportions of lymphocytes. These were located in the blood-filled, dilated sinuses of the marrow. No foci of necrosis or degeneration were found.

Histological examination of gastrointestinal tissues from the above animals revealed a general hyperemia. Actual lesions were confined to ileum and colon. Tips of villi were distended by enlarged vessels and plasma transudate under epithelial linings. Dilated capillaries in sub-mucosa, adjacent to muscular coats and surrounding crypts, contained increased numbers of leukocytes. Migration of leukocytes into the lumen of the crypts was evident, forming plugs with desquamated epithelial cells. In crypt-epithelium mitosis was almost absent and enlargement of nucleus and cytoplasm as well as desquamation with pyknosis was observed.

Animals receiving repeated injections for 17 to 30 days are listed in Table II (group I and II). Group I received either 20 mg/kg/day during the first three weeks followed by injections of 40 mg/kg/day for the remainder of the period, or 40 mg/kg/day throughout. Group II was treated in similar fashion but, in addition, was given prior to sacrifice 2 or 3 final daily doses of 79 mg/kg. Significant weight loss was observed only in rats of group II after receiving 79 mg/kg/day.

In spite of prolonged treatment animals of Group I and II were less affected than "acute" rats. However, the effect of diaminopurine on erythropoiesis was readily discernible in animals receiving the extended treatment. Depletion of nucleated red cells in marrow and reticulocytopenia were significant (I and II, Table II). Anemia also occurred in 2 of each group. No significant alteration occurred in erythrocyte-volume or content of hemoglobin. Only 6 of 8 animals in group I revealed depletion of nucleated cells in bone marrow, approximating 50%, while reduction in group II was no greater than that found in "acute" rats. Increased proportions of lymphocytes appeared only in marrows of group II (Table II). A predominance of basophilia in nucleated red cells and hypersegmentation of polymorphonuclears were evident in marrows of both groups. Gastrointestinal lesions were not found microscopically in group I and were confined to minimal epithelial changes in crypts of colon and ileum in 3 of 8 rats in Group II.

Histological examination of other organs of animals in the 3 groups described above, revealed no significant lesions but for a moderate reduction of lymphoid tissue in thymus, spleen, mesenteric nodes, and intestinal wall. Reduction of myeloid tissues in spleen was also noted in animals with depleted marrows.

Course of intoxication in dogs. The administration of 40 or 50 mg/kg in dogs 1 to 4 (Table III) produced an immediate, transient hyperpnea. Vomiting occurred at 3 hours and continued profusely until death. Food was refused after the first day. Hemorrhagic diarrhea appeared within 24 hours and increased in severity progressively. During the course of intoxication animals lost weight (Table III) and developed severe upsets in fluid and electrolyte excretion. Thus, dogs 3 and 4 excreted less than 500 cc/day of urine prior to treatment while during 54 hours of intoxication the former produced 8 l of fluid (urine, vomitus, and loose stool) containing 184 m eq Cl^- and the latter, 5 l containing 247 m eq Cl^-. Intake of water *ad libitum* was increased in proportion. Dogs 1 and 2 succumbed after 40 hours; dog 4, after 54 hours; and dog 3 was sacrificed when moribund at 54 hours.

The remaining dogs (5 to 8, Table III) receiving lower daily doses of diaminopurine evidenced fewer signs of intoxication. Dogs 6, 7, and 8 lost weight. Occasional vomiting and diarrhea without blood occurred only in the last few days prior to their sacrifice (4, 29 and 31 days respectively). Dog 5 recovered after a moderate weight-loss (Table III and Fig. 1).

The course of lethal intoxication in dogs receiving large doses of diaminopurine can be of shorter duration than in animals given supralethal doses of 4-amino-PGA. Protracted emesis is not a feature of intoxication with the latter agent. Moreover, diarrhea does not appear in dogs poisoned with 4-amino-

TABLE III.
Hematological Data from Dogs Injected Intravenously with 2, 6 Diaminopurine.

				Peripheral blood				Sternal bone marrow					
Dog	Day	Daily dosage, mg/kg	Wt, kg	HCRIT, %	RETIC, %	GRCYT, 10^3/mm^3	LYCYT, 10^3/mm^3	NCC, 10^3/mm^3	N GRCYT, %	EOS GRCYT, %	MYEL, %	LYCYT, %	ERYTH, %
1	0	50	16.3	43.0	0	17.3	.7	170	32	2	14	8	44
	1		14.6	53.5	0	17.6	0	36	90	1	1	7	1
2	0	50	15.7	54.0	pr	10.6	1.6	245	63	6	18	2	11
	1		14.7	62.5	0	16.0	.2	25	95	1	2	1	1
3	0	40	19.2	41.5	2.2	18.4	1.0		46	4	23	2	25
	2		16.8	59.0	0	5.8	3.7		72	2	3	14	9
4	0	40	19.9	39.0	0	36.0	3.6		49	5	19	4	23
	2		16.2	58.5	.3	38.2	0		97	1	pr	0	2
5	0	20	13.6	44.0	.3	16.7	4.2	45	54	2	12	21	11
	4		13.3	41.5	0	9.8	1.1	10	85	0	5	10	0
6	0	20	10.6	41.0	.6	20.4	1.3	80	67	1	13	8	11
	4		8.2	51.0	0	11.2	.3	14	87	0	8	4	1
7	0	8	18.3	40.0	.7	9.0	1.2	65*	37	3	14	16	30
	18	20	16.7	37.0	0	15.6	.5	80†	70	5	12	8	5
	28		13.8	40.5	0	.1	.1	7	9	3	83	3	2
8	0	8	19.0	48.0	.6	10.8	.8	220*	41	3	8	5	43
	18	20	17.6	45.0	0	7.6	.2	31†	61	7	21	8	3
	28		14.1	50.5	0	5.9	.1	15	80	5	12	2	1

* Value at 6 days.
† Value at 21 days.

HCRIT, hematocrit.
NCC, nucleated cell count.

pr, present, less than 0.5%.
Other abbreviations as in Table II.

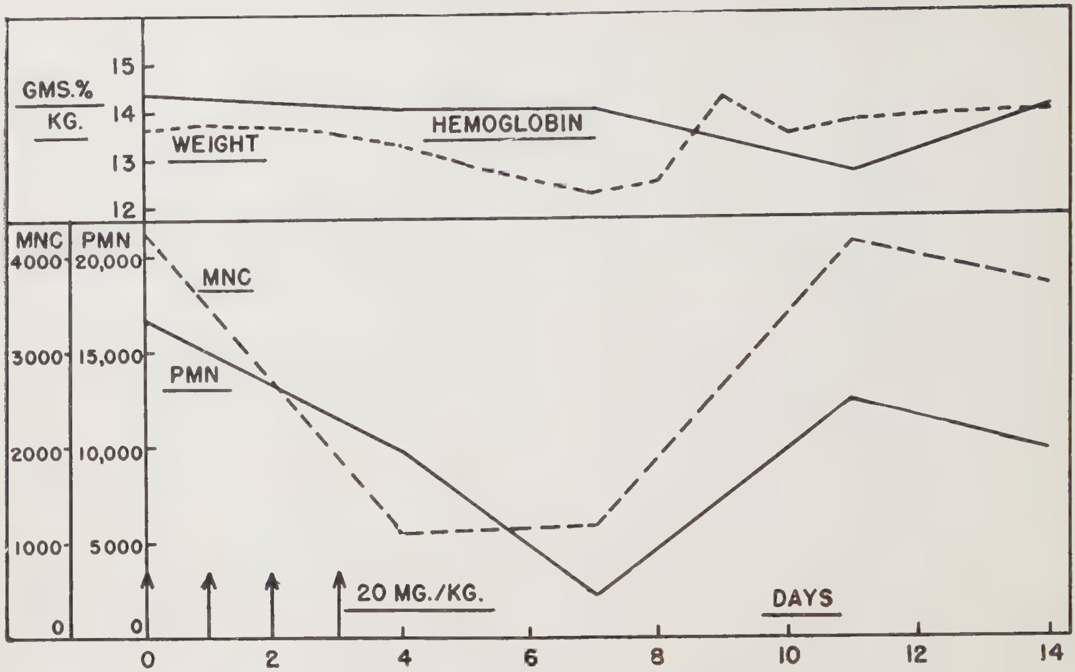

FIG. 1.

Changes in weight and peripheral blood of dog 5, following repeated doses of diaminopurine. MNC, lymphocytes and monocytes/mm^3; PMN, granulocytes/mm^3.

PGA until after 48 hours and death does not occur before 72 hours.[2]

Lesions in dogs. At autopsy all dogs appeared dehydrated. Bone marrow in sternum, ribs, and vertebrae appeared dry and sparse; femoral marrow, brownish and fatty. The intestinal tract of dogs 1 to 4 was edematous from duodenum to anus. Petechial hemorrhages were visible in ileum and colon and extensive around the anus. The contents of the gut were fluid and blood-stained. Dogs 6, 7 and 8 showed only edema of the intestinal mucosa.

Findings in peripheral blood are noted in Table III. Hemoconcentration developed in dogs 1 to 4 and 6 and might have been masked in dogs 7 and 8 by coexisting anemia. No changes in erythrocyte-volume or hemoglobin-content were noted. Reticulocytes and lymphocytes decreased in numbers in most animals. Granulocytopenia developed in dogs 5 to 8, which had been observed for more than 48 hours. Details of changes in peripheral blood of dog 5 are noted in Fig. 1.

Bone marrow was studied with aspirations from sternum and both iliac crests as well as with tissue-blocks taken at autopsy from ribs and femur. Only values from sternal marrow are reported in Table III since they corresponded closely to samples from other sites. A reduction of total nucleated cells was noted in all animals when measured. This observation was confirmed by study of smears and sections which contained only scattered islands of hematopoietic tissues. The outstanding feature of the depletion was the marked reduction of erythropoietic tissues. Surviving cells of the erythroid series included the naturally occurring erythrocytes with nuclear remnants, normoblasts, and erythroblasts. Although myeloid cells shared in the general depletion, all dogs with one exception evidenced increased percentages of polymorphonuclear granulocytes with relative decreases of immature myeloid forms. The marrow of the exceptional dog, 7, contained at 28 days, a high percentage of myelocytes in association with an intercurrent infection. Details of marrow findings in dog 5 are shown in Fig. 2.

Histological study of the gastrointestinal

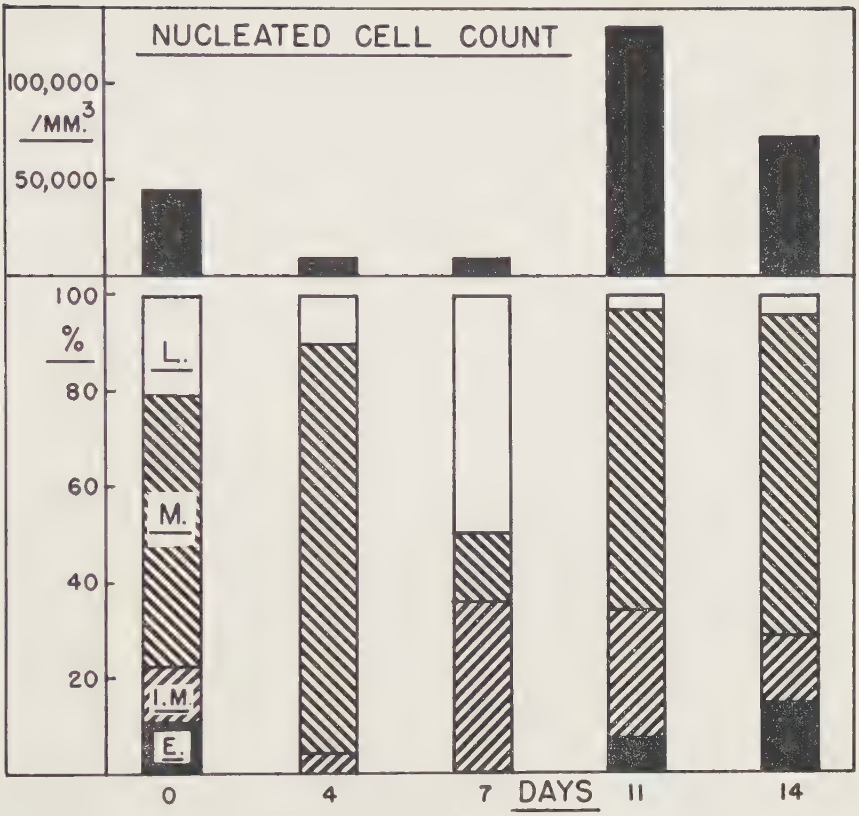

FIG. 2.

Changes in cell-count and differential count of nucleated cells from sternum of dog 5, following repeated doses of diaminopurine. L., lymphocytes; M., polymorphonuclears; IM., myeloblasts, myelocytes and metamyelocytes; E., red cells.

tract of dogs 1 to 4 revealed changes similar to those described above in rats receiving 100 mg/kg/day of diaminopurine. In addition, superficial ulcers of mucosa and extensive matting of villi occurred in ileum and colon. The remaining animals showed only edema and hyperemia of the intestinal mucosa. Changes in other tissues resembled those seen in rats.

Discussion. Since other purines such as caffeine[10] or adenine[11] are known to cause a variety of acute pharmacological actions, it was not unexpected that large doses of 2,6-diaminopurine should result in death within a few hours after administration. Indeed, the gross behavior of rats and mice is similar following equivalent doses of either 2,6-diaminopurine or adenine (6-aminopurine) greater than 500 mg/kg.[12] On the other hand 4-amino-PGA is inert from the standpoint of acute pharmacological effects.[1,2] At present it does not appear pertinent to compare diaminopurine and 4-amino-PGA on the basis of such actions which bear no known relationship to metabolite-antagonism. More appropriate is a comparison of lesions in intestines and bone marrow caused by both agents. Their effects on mucosa of ileum and colon of rats and dogs cannot be differentiated by histological studies. The depletion of hematopoietic tissues following effective doses of either agent can be of similar severity. However, certain features of the disturbance in hematopoiesis caused by 4-amino-PGA in dogs are unique such as the appearance of abnormal mitosis and "nuclear explosions" in normoblasts, alteration of the nuclear pattern of mature and primitive erythroid cells and formation of cells resembling megaloblasts, and production of bizarre, giant metamyelocytes.[2] Similar pathological cells have not been observed in dogs receiving diaminopurine. Therefore, it cannot be inferred that the agent acts as an antagonist of PGA in the metabolism of hematopoietic tissues.

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Summary. The administration of large doses of 2,6-diaminopurine in mice and rats can result in death within a few hours. In dogs, large doses caused protracted vomiting, hemorrhagic diarrhea, dehydration and death within 48 hours. Doses permitting survival of rats and dogs for 24 hours or longer produced depletion of bone marrow and damage to epithelium of colon and ileum. The course of intoxication and changes in bone marrow permit a differentiation between the actions of 2,6-diaminopurine and a folic acid-antagonist like 4-aminopteroylglutamic acid.

Received October 5, 1949. P.S.E.B.M., 1949, **72**.

Effect of Mercury on Renal Tubular Transfer of p-Aminohippurate and Glucose in Man. (17449)

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The depressant effect of intravenously administered salyrgan (mersalyl) on the maximum renal tubular secretion of p-aminohippurate (Tm_{PAH}) in man has been demonstrated.[1,2] The inability of the mercurials, salyrgan and meralluride (mercuhydrin), to depress the Tm_{PAH} in the dog has also been shown.[2,3] That the maximum tubular reabsorption of glucose (Tm_G) in the dog is unaffected by mercury chloride or meralluride in dosages of 4 mg of bound mercury per kilogram of body weight has been established recently.[3] With regard to the Tm_G in man, Weston *et al.*[4] have reported that the intravenous administration of 2 cc of mercuzanthin or thiomerin, "immediately after several ten minute control periods generally resulted in a 40-80% decrease."

Thus the current concept is that intravenously administered mercurials depress the renal tubular transfer systems for PAH and glucose in man but leave unaffected these systems in the dog. It is the purpose of this study to re-examine the effect of a mercurial, mercuzanthin, on the Tm_{PAH} and Tm_G in man.

Method. Ten fasting male subjects were used in the Tm_G observations. Five of the 10 subjects used for the Tm_G observations plus an additional subject were used in the Tm_{PAH} determinations. All of the subjects were free of demonstrable renal dysfunction. The study pattern was the same for all of the subjects. Using the saturation methods outlined by Goldring and Chaisis[5] 3 control urine collection periods, each of 12 minutes duration, were obtained. Two cc of mercuzanthin* were injected intravenously at the end of the third control period. Immediately following the third period, 5 consecutive 20

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* One cc of mercuzanthin represents 135 mg of the sodium salt of b-methoxy-hydroxy-mercuri-propylamide of cyclopentane dicarboxylic acid equivalent to 39 mg of mercury and 35 mg of anhydrous theophylline.

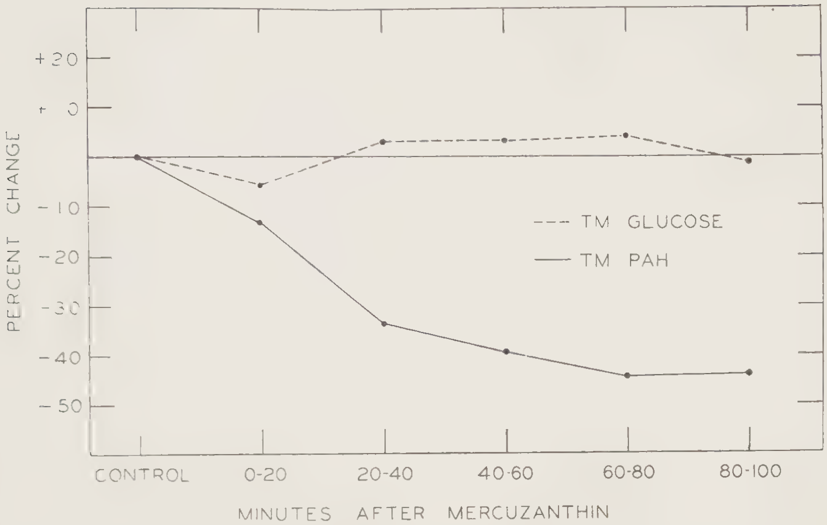

FIG. 1.

The individual points represent the mean per cent change in Tm_G of 10 subjects and in Tm_{PAH} of 6 subjects for 5 consecutive 20 minute urine collection periods following the intravenous administration of 78 mg of organically bound mercury. The control value is based on the mean of three 12 minute urine collection periods for each subject.

minute urine collection periods were obtained. The total time following injection of the mercury to the end of the last urine collection period was 100 minutes. Inulin, used in measuring the glomerular filtration rate, was determined using Harrison's modification of the method of Alving.[6] PAH determinations, using diluted urine and cadmium sulfate filtrates of plasma, were made by the method of Bratton and Marshall.[7] Glucose determinations were made according to the method of Nelson.[8]

Results. When the values for the group were averaged for each period, the maximum depression of Tm_{PAH} was 44.5% of the control value and occurred during the urine collection period from 60-80 minutes following the administration of mercuzanthin (Fig. 1). The mean Tm_{PAH} values decreased from 73.3 mg/min to 44.9 mg/min following the administration of mercury (Table I). This difference is significant ($N = 6$, $t = 11.05$, $P < .01$). In the case of the Tm_G observations, no significant difference was noted between the mean values of the pre- and post-mercurial observations ($N = 10$, $t = .275$, $P > .70$, Table I). The first postmercurial urine collection period (0-20 minutes) was not used in calculation of the mean for either Tm_G or Tm_{PAH} because of the possible effect of the theophylline base on the renal tubular function. The apparent decrease in Tm_G for the first post-mercurial urine collection period (0-20 minutes) is not significant.

Comment. The renal tubular reabsorption of glucose is generally believed to be effected through a phosphorylation mechanism.[9] Assuming that this is the mechanism operative in glucose reabsorption by the renal tubules, mercury must have been ineffective, at least in the amounts used in this study, in blocking the activity of this system. This belief finds an interesting parallel in the studies of Hepler and Simonds.[10] Using mercury chloride, these investigators demonstrated that glycosuria could not be induced in dogs unless the animals showed a 4 + albuminuria and marked renal tubular necrosis. The observa-

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TABLE I.
Changes in Tm$_{\mathrm{G}}$ and Tm$_{\mathrm{PAH}}$ Following Mercury Administration.

	Tm$_{\mathrm{G}}$, mg glucose/1.73 sq. m/min.			Tm$_{\mathrm{PAH}}$, mg PAH/1.73 sq. m/min.		
Age, yr	Control	Post-mercury	% change	Control	Post-mercury	% change
53	296	316	+ 6.8	98.3	58.3	—40.7
57	206	191	— 7.3	—	—	—
44	254	270	+ 6.3	52.3	23.3	—55.4
59	198	200	+ 1.0	—	—	—
65	248	248	± 0.0	50.6	24.9	—50.8
67	271	312	+15.1	—	—	—
53	377	361	— 4.2	83.2	54.3	—34.7
62	252	221	—12.3	—	—	—
61	224	267	+19.2	83.4	61.5	—26.3
48	246	238	— 3.3	—	—	—
69	—	—	—	71.9	47.2	—34.4
			Mean			
58	257.2	262.5	+ 2.1	73.3	44.9	—40.4

Control values represent the mean of 3 twelve minute urine collection periods. Post-mercury values represent the mean of 4 twenty minute urine collection periods, extending from 20 to 100 minutes following the injection of mercuzanthin.

tion that the Tm$_{\mathrm{PAH}}$ is diminished while the Tm$_{\mathrm{G}}$ is unchanged following the administration of mercury strongly implies that the mechanisms involved in PAH and glucose transfer are different. However, in conjunction with this belief, it is interesting to note that the simultaneous measurement of Tm$_{\mathrm{PAH}}$ and Tm$_{\mathrm{G}}$ in the dog results frequently in a depression of the former and occasionally in a depression of the latter.[11,12] In man the simultaneous determination of Tm$_{\mathrm{PAH}}$ and Tm$_{\mathrm{G}}$ may result in a depression of the former and an apparent increase in the latter.[13] At present the observed depressions are presumed to be a result of competition for available energy between the PAH and glucose transfer systems with the latter being favored.[11]

Summary. The intravenous administration of 78 mg of organically bound mercury (2 cc mercuzanthin) causes a significant decrease in the Tm$_{\mathrm{PAH}}$ but no significant change in the Tm$_{\mathrm{G}}$ in man. It is concluded that under the conditions of this study mercury had no effect on the renal tubular glucose transfer system in man.

The technical assistance of Mrs. Eleanore Roach, Mrs. Elsie Beard, Miss Margaret McCollum, Mr. Charles Punte, and Mr. Robert Faid is gratefully acknowledged.

Received October 4, 1949. P.S.E.B.M., 1949, **72**.

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Destruction of Red Blood Cells. VII. Apparent Autosensitization to Dog Red Blood Cells.* (17450)

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In an occasional case of atypical hemolytic anemia a serum agglutinin active against the patient's own red blood cells has been found.[1] This may be merely the result of the presence of a serum substance with entirely fortuitous properties resembling in activity the seemingly nonspecific agglutination of red blood cells by antiserum developed in rabbits against Type 14 pneumococcus[2] or by certain viruses.[3] There are, however, two possible strictly immunological explanations for the presence of such an autoagglutinin: 1. The antibody (autoagglutinin) could be formed in response to some antigen in the red blood cell, or 2. the antibody could be formed for some other antigen but might only incidentally possess the capacity to react with a receptor in the blood cell.

That an animal can produce antibody for components of tissues of the same species has been amply demonstrated.[4] Hektoen and Schulhof,[5] after injecting rabbits intravenously with solutions of rabbit thyroglobulin, demonstrated serum precipitins for rabbit thyroglobulin in a titer as high as 1:10,000. Such sera did not react with the thyroglobulin of beef, dog, human, monkey, rat, sheep or swine. Several workers[6,7] have shown that formalized rabbit serum is antigenic for rabbits. Furthermore, rabbit brain extracts (containing lipoids)[8] and rabbit fibrinogen[9] stimulate the formation in rabbits of complement-fixing antibodies and precipitins. Schwentker and Comploier[10] and the Caveltis[11] have demonstrated the formation of complement-fixing antibody for homologous renal tissue.

Such observations suggested that an animal might be sensitized experimentally to its own red blood cells. If such sensitization were demonstrated it would support the explanation that in certain instances of acquired hemolytic anemia in man the serum autoagglutinins may be formed in response to an antigen in red blood cells. Therefore, the following studies were made.

Materials and Methods. Four female mongrel dogs each weighing approximately 25 lb were maintained on a diet of horse meat and commercial preparations of dog food supplemented with 1.2 g of ferrous sulfate a day. Red blood cells, hemoglobin concentration, hematocrit, icterus index, and reticulocyte and white blood cells in oxalated samples of venous blood were determined by conventional methods on the average of every 10 days. Repeated determinations of osmotic and mechanical fragility of the red blood cells were made by modifications of technics previously described.[12,13] Serum complement titrations, complement fixation, and cold agglutinin and warm agglutinin titers were followed periodic-

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* This investigation was aided in part by a grant from the John and Mary R. Markle Foundation.

† Research Fellow of the American College of Physicians.

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ally according to standard technics.[14,15] Serum from rabbits previously injected repeatedly with normal dog serum was heated at 56°C for 30 minutes and employed in a manner similar to that described by Coombs *et al.*[16] Thus, equal aliquots of saline suspensions of washed dog cells were incubated for one hour at 37°C with the anti-dog serum rabbit serum, centrifuged at about 300 rpm for 2-3 minutes, resuspended by gentle agitation and examined microscopically under a cover slip for agglutination.

Procedures and Results. Several methods were employed. The red blood cells from 10 cc of defibrinated blood were frozen, thawed and then incubated in 4 cc of streptococcal toxin containing 400,000 skin test doses for 24 hours at 37°C. The suspension was then injected intraperitoneally into the dog from which the cells had been obtained. Fourteen such injections were made in 2 dogs at 2 day intervals. During the period of injection and for the 18 following days no significant alterations of results in the laboratory tests listed above were found.

In the study of 2 other dogs red blood cells from 10 cc of defibrinated blood were incubated 24 hours at 37°C after being inoculated with 6 to 24 hour broth culture of hemolytic *Staphylococcus aureus* (Costello strain). The blood culture was then filtered through a Seitz filter and frozen for 24-48 hours before being used. After thawing, the filtrate was injected intraperitoneally into the dog from which the cells had been obtained. To 1 dog 10 such injections were given and to another dog 11 injections at 48-72 hour intervals. During the period of injection and the following 17 days no significant alterations of results in the laboratory tests listed above occurred. One dog showed fluctuations in mechanical fragility of its red blood cells from 3.2 to 50% unassociated with other changes in the results of laboratory tests listed. As marked fluctuations in mechanical fragility of apparently normal dog cells sporadically occur[17] such an isolated observation was not considered indicative of autosensitization.

Seventeen to 18 days after the previous experiments each of the 4 dogs was given a single subcutaneous injection of a modified Freund antigen[18] consisting of a mixture of 1 cc of its own washed, packed red blood cells, 1 cc of mineral oil[‡], 0.2 cc of a lanolin-like substance[§] and 1 mg of heat killed *Mycobacteria tuberculosis* (H37Rv strain).[‖] Over a period of 6 weeks following the injection no changes were noted in osmotic fragility or complement titer and no development of cold or of warm agglutinins or of serum complement-fixing antibodies occurred. The washed red blood cells did not clump in anti-dog serum rabbit serum. The mechanical fragility of the cells from 2 of the dogs fluctuated in an unpredictable manner. However, in light of previous experience[17] this was not considered indicative of autosensitization.

Six weeks after the injection of the above described modified Freund antigen a second similar preparation was made with the red blood cells of each dog. The mixture was then heated at 57°C for 2½ hours, cooled, added to 2 cc of fresh pig serum and injected immediately subcutaneously. Again there were no changes in 3 of the dogs as tested by the laboratory procedures listed above. However, in 8 observations made from the 25th to the 35th post-injection day the washed red blood cells from the fourth dog showed definite agglutination when suspended in anti-dog serum rabbit serum. On the 36th, 37th, 38th and on subsequent post-injection days the cells were found non-agglutinable by such a procedure. During the period in

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[‡] The brand of mineral oil used was Bayol F, supplied by the Standard Oil Company of New Jersey.

[§] The lanolin-like substance was Falba, the trade name for a preparation of Pfalz and Bauer, Inc., New York.

[‖] Supplied through the courtesy of William Steenken, Jr.

which the washed red blood cells were agglutinated by anti-dog serum no anemia or increase in reticulocytes or icterus index appeared. The osmotic fragility of the red blood cells remained normal. No cold or warm agglutinins were demonstrable. The complement titer of the serum (1:32) did not fall; and no serum complement-fixing antibody for the dog's own red blood cells was demonstrable. The mechanical fragility of the red blood cells ranged from 3.1 to 6.1%; values considered by Shen[17] to be normal for dog red blood cells.

Summary and Conclusions. Several attempts were made to sensitize 4 dogs to their own red blood cells. Three dogs showed no definite evidence of autosensitization. Although 2 of these 3 dogs showed occasional marked fluctuation in mechanical fragility unaccompanied by other changes during these studies this was considered to be due to technical difficulties. However, for a short period in one dog, 25 days after the injection of a modified Freund antigen, there was agglutination of washed red blood cells when they were suspended in anti-dog serum rabbit serum. During this period of apparent autosensitization there was no anemia, reticulocytosis or jaundice. The red blood cells showed no alteration in osmotic or mechanical fragility. There was no fall in complement titer. Cold and warm agglutinins did not appear. There was no evidence of a serum complement-fixing antibody for the dog's own red blood cells. Such apparent autosensitization supports the explanation that in certain instances of acquired hemolytic anemia in man serum autoagglutinins may be formed in response to an antigen in red blood cells.

Received October 10, 1949. P.S.E.B.M., 1949, **72**.

The Appearance of Acetylcholine During Normal Labor. (17451)

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In our earlier studies on the acetylcholine (Ach) of the human placenta,[1] blood from pregnant women was not available for investigation. In consideration of the potent hydrolytic action of the blood cholinesterase, it was inferred that Ach of human placenta does not appear to reach the maternal circulation in any noticeable quantity.

Opportunity to restudy this problem has not presented itself until lately. On the basis of an analysis of 288 blood samples of 152 cases during pregnancy and at different stages of labor, our previous impression has to be modified.

Four cc of blood were obtained each time and poured into 2 volumes of 95% pure alcohol. The mixture was thoroughly ground, diluted with 2 volumes of saline and centrifuged. The supernatant fluid was measured, and usually 0.5 cc or less was used for assay. Alcohol-free extract was also prepared by evaporating 5 cc to dryness in room (23-30°) and re-taking up with 5 cc of frog Ringer solution. The extracts were assayed on the toad's rectus test which depends on the principle of equal potentiation of Ach standard and the unknown by eserine. So if the matched ratio is identical before and after eserine, it is taken as Ach. Otherwise, Ach is considered to be nil.

Table I gives results showing a gradual appearance of Ach toward the second trimester of gestation, an apparent increase of Ach with the onset of labor, and a disappearance beginning 48 hours after labor.

The blood of 10 non-pregnant women and

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10 male individuals showed no evidence of Ach by the same test. Trichloracetic extract prepared according to Chang and Gaddum[2] gave results of the same order as that of alcohol extract.

Although alcohol has a potentiating action on Ach,[3,4] it did not appear to influence the results of our test under the routine precautions taken. In 25 trials to study this point, the alcoholic extracts gave an average of 1.355 ± 0.090 γ/cc, while the corresponding alcohol free extracts gave an average of 1.181 ± 0.105 γ/cc, the difference being 0.174 ± 0.139.

From the studies made on placental tissue and perfused placenta,[5] it was suggested that Ach may play a local role in the normal mechanism of labor. Any theoretical deduction made from studies on a tissue which has been discharged as being of no more use to the body is to be guarded against. By demonstrating an increased blood Ach toward term and with the onset of labor, our suggestion that Ach may play a role in the normal mechanism of labor is further strengthened.

In view of this finding of blood Ach, the suggestion of using eserine for induction of labor[5] deserves a trial which is in progress.

Summary. In a study of 288 samples of blood from 152 cases, a gradual appearance of Ach toward the 2nd trimester of pregnancy, an increase of Ach with the onset of labor, and a disappearance 48 hours or more after labor were demonstrated. Blood of 20 normal subjects of both sexes showed no evidence of Ach with the same test.

TABLE I.
Increase in Acetylcholine of Blood During Pregnancy.

	During pregnancy			Before onset of labor	During labor		After labor	
	1st trimester	2nd trimester	3rd trimester		Early stage	Late stage	Before 48°	After 48°
Total No. of samples	26	30	23	26	44	66	47	26
Samples without Ach: No.	25	21	17	10	8	17	34	26
%	96.2	70.0	73.9	38.5	18.2	27.8	72.3	100
Samples with Ach: No.	1	9	6	16	36	49	13	0
%	3.8	30.0	26.1	61.5	81.8	72.2	27.7	0
Range of Ach in blood, γ/cc	1.61	0.69-3.06	0.71-1.94	0.73-4.70	1.02-7.59	0.71-6.81	0.67-6.21	—
Average	—	1.871 ± 0.220	1.332 ± 0.164	2.586 ± 0.324	3.089 ± 0.286	2.387 ± 0.199	2.929 ± 0.450	—

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Received August 12, 1949. P.S.E.B.M., 1949, **72**.

Probable Absence of Citrulline from Proteins. (17452)

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Several early investigators noted that carbon dioxide is one of the products of the acid hydrolysis of proteins. Dunn[1] found that as much as 0.81% of casein could be accounted for as carbon dioxide after 30 hours of acid hydrolysis. It has been generally supposed that this carbon dioxide is bound as a ureide in the protein molecule and the exact type of binding is uncertain. Fearon's[2] diacetyl monoxime test for ureide compounds is positive for many proteins. Wada[3] reported the isolation of citrulline from tryptic digests of casein, but this work has never been repeated. Since certain types of bacteria can convert arginine to citrulline, it is possible that Wada's citrulline may have been formed by bacterial action during the enzymatic hydrolysis.

This investigation was undertaken in order to obtain additional evidence as to the nature of the ureide groupings in proteins.

Methods and results. The proteins used in this study were all prepared in the laboratory. Casein was prepared according to the method of Van Slyke and Baker,[4] the crystalline globulins of watermelon seed and pumpkin seed by the method of Vickery,[5] arachin by the method of Johns and Jones,[6] amandin by the method of Osborne,[7] and myosin by the method of Dainty, Kleinzeller, *et al.*[8]

The 2 crystalline globulins were recrystallized twice after the initial isolation, and the arachin and amandin were twice dissolved and precipitated by dialysis after the initial isolation. The casein and myosin were used as originally isolated.

After preparation the proteins were air dried until completely equilibrated with regard to moisture content. They were analyzed for moisture and ash, and for nitrogen by the Kjeldahl method using mercury as the catalyst. The results of these analyses are given in Table I.

These proteins gave moderate to marked positive tests for ureide groups when tested under the conditions of Archibald's[9] citrulline determination. After they had been hydrolyzed for 16 hours or longer with 6N hydrochloric acid, none of them gave positive tests. It was thought that this might be due to destruction of any citrulline contained in the proteins during the hydrolysis, so the destruction of citrulline alone under the conditions employed for hydrolysis of the proteins was studied. One ml samples of solutions of citrulline (about 1 mg/ml) in 6N hydrochloric acid were sealed in small glass bomb tubes and heated in the oven at 110°C. At intervals samples were removed and cooled, and their citrulline content was determined by Archibald's method. Under these conditions citrulline is surprisingly resistant to acid hydrolysis; it is destroyed by a first order reaction whose half-life is approximately 26 to 28 hours, as is seen in Fig. 1. This is not conclusive evidence for the absence of citrulline from proteins since it is well known that cystine and threonine are much more easily destroyed when in peptide combination than when free. Other experiments in which paper chromatography was employed to identify the products of hydrolysis of citrulline showed that under these conditions ornithine was the only product formed in detectable amounts.

Since Wada's isolation of citrulline was

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TABLE I.
Analysis of Protein Samples.

Protein	Moisture, %	Ash, %	N, %	N, % M and A free
Casein	10.36	0.37	13.78	15.44
Myosin	10.04	1.03	15.03	16.90
Arachin	9.44	2.86	16.01	18.26
Amandin	9.38	0.43	17.45	19.35
Pumpkin seed globulin	10.23	0.15	16.47	18.38
Watermelon seed globulin	9.31	0.06	16.77	18.50

done from a tryptic digest of casein it was decided to determine the amounts of citrulline present in enzymatic hydrolysates of the 6 proteins. Seven 1 g samples of each of the proteins were weighed out into 125 ml Erlenmeyer flasks. About 1 g of precipitated calcium carbonate was added to each flask, together with 50 ml of water. The samples were allowed to stand for 24 hours in the refrigerator in order for the protein to become thoroughly wetted. Ten mg of Difco, 1/110 trypsin were then added to each flask and to each of 7 enzyme blanks containing calcium carbonate and water. A known amount of citrulline was added to 3 of the samples of each protein and to 3 enzyme blanks; about 1 ml of xylene was added to each flask and all samples were incubated at 37°C. At intervals samples were withdrawn from one enzyme blank and from one sample (containing no added citrulline) of each protein and titrated by Northrup's[10] formol titration, modified in that the end points were determined with the aid of a Beckman pH meter. When the amino nitrogen contents had become constant in the pilot flasks an additional 10 mg of trypsin and 1 ml of an active Waldschmidt-Leitz[11] preparation of erepsin were added to each flask. Digestion and determination of the amino nitrogen in the pilot flask was continued; and when the amino nitrogen had become again constant an additional ml of erepsin was added to each flask. After one week's additional digestion the samples were removed from the incubator, filtered into 100 ml volumetric flasks, and made up to volume with the washings. Aliquots of these digests were taken for determination of total amino nitrogen content and of total ureide content. The results are shown in Table II. In each case a correction has been made for both amino nitrogen and citrulline content by subtracting the value found in the appropriate blank from that found in the protein digest. In no case was more than 0.09% of the protein accounted for as "citrulline" by the colorimetric determination; however, in all runs in which citrulline was added to the digests the recoveries in terms of color intensities were extraordinarily high, *i.e.*, the observed colorimetric values corresponded to from 300 to 500% of the amounts added. It was thought that a peptide with enhanced chromogenicity might have been formed during hydrolysis, but similarly high "recoveries" were obtained when the citrulline was added to the digests immediately before determination and even when the digests had been heated for half an hour on a boiling water bath to inactivate any residual enzyme.

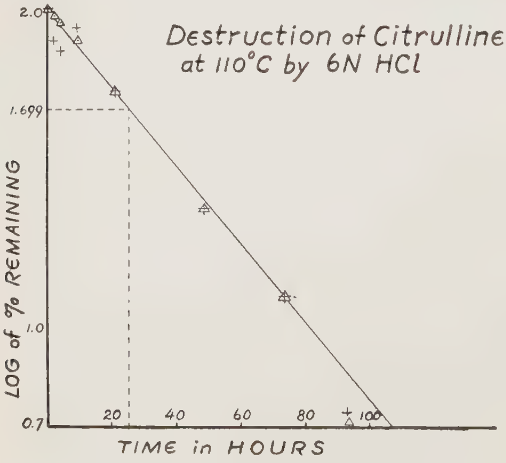

FIG. 1.

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TABLE II. Citrulline Content of and "Recovery" from Protein Digests.

Protein	% N titrable as NH_2–N after acid hydrolysis (A)	% N titrable as NH_2–N after enzyme hydrolysis (B)	% digestion (100 B/A) (C)	% Citrulline in protein (M and A free) (D)	Citrulline added to digest, mg/100 ml (E)	Citrulline found, mg/100 ml (F)	% recovery (G)
Casein	11.80	8.01	67.8	0.093	4.675*	13.66	271
					3.260†	12.82	391‡
Myosin	12.76	8.79	69.0	0.089	4.675*	15.00	299
					3.260†	10.70	299§
Arachin	12.36	8.03	65.0	0.044	4.675*	15.56	320
					3.260†	11.20	336‡
Amandin	13.27	5.02	37.9	0.080	4.675*	18.14	368
					3.260†	15.08	435‡
Pumpkin seed globulin	12.01	8.13	67.6	0.072	4.675*	18.66	381
					3.260†	13.51	388§
Watermelon seed globulin	11.75	8.12	69.1	0.066	4.675*	17.02	346
					3.260†	11.02	316‡

* Present throughout digestion.
† 1 ml citrulline solution diluted to 25 ml with digest.
‡ Not boiled.
§ Boiled.

Since these extraordinarily high "recoveries" were not obtained in the enzyme blank, substances other than the proteins or their digestion products are eliminated as a cause for the high "recovery." Amino acids cannot cause this effect since an acid hydrolysate of casein added to citrulline and put through the colorimetric determination of citrulline did not give high results. Similar but less markedly high "recoveries" were obtained when commercial peptones were added to citrulline solutions, so that it would appear that the presence of partial digestion products of the proteins in a citrulline solution markedly enhances the chromogenicity of the citrulline in the diacetyl monoxime reaction.

Discussion. Although the results obtained in this study are not absolutely conclusive in demonstrating that citrulline is not present in proteins, they nevertheless strongly indicate that it is absent or present only in very small amounts in the 6 proteins studied. Citrulline might be more readily destroyed by acid when in peptide combination than when in the free state, but unless this difference were very great, small amounts of it should survive acid hydrolysis. Acid hydrolysates were completely negative for citrulline by the diacetyl monoxime reaction. Furthermore, if significant amounts of citrulline were present, it should have been recovered in the enzymatic digests. The exact nature of the ureide compound present cannot be determined from these results. The virtual elimination of citrulline as a possibility enhances the probability that the original ureide may be either a cyclic type of structure or more probably simply carbamic acid present as a constituent of the peptide chain and hydrolyzed and spontaneously decomposed during enzymatic hydrolysis.

Summary. 1. Casein, amandin, arachin, myosin, and the crystalline globulins of pumpkin seed and watermelon seed give marked to moderately strong diacetyl monoxime reactions for citrulline when tested directly but their acid hydrolysates give completely negative results.

2. Citrulline itself is relatively slowly destroyed by treatment with 6N hydrochloric acid at 110°C.

3. Only very small amounts of ureide com-

pounds are found in enzymatic hydrolysates of the 6 proteins; however, when citrulline is added to such hydrolysates, its "recovery" in terms of colorimetric units corresponds to several times the theoretical value. The reason for this anomalous result has yet to be determined.

4. It is probable that citrulline is either absent or present only in minimal quantities in these proteins.

This work was supported in part by a grant (INSTR. 23) from the American Cancer Society. The authors wish to thank Miss Jane Price for the nitrogen analyses of the protein preparations.

Received Sept. 10, 1949. P.S.E.B.M., 1949, **72**.

Comparative Study of Lipids in Whole Carcasses of Arctic and Non-Arctic Fish.* (17453)

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Information concerning lipid content and metabolism in cold-blooded animals is relatively meager.[1] Concerning these matters in Arctic poikilotherms, information is non-existent. A survey was, therefore, made of the lipid content of whole fish, *Pygosteus pungitius*,[2] the Arctic 9-spined stickleback. The results were evaluated in comparison with similar analyses made on the common guppy *(Girardinus guppyi)*.

Material and methods. Whole sticklebacks or guppies were carefully weighed and then dropped into a mixture consisting of 3 parts of pure ethanol and 1 part benzine. After 24 hours the fish were ground with sand and the lipids extracted according to Bloor's method.[1] Total fatty acids were estimated using the method of Bloor as modified by Snell.[3] Lipid phosphorus was estimated by a modified Youngburg technic;[4] from the latter results phospholipids were calculated as lecithin. Measurements of cholesterol were based on methods developed by Bloor.[5] Since all the procedures are colorimetric, a Klett-Summerson photometer was used to measure the various color intensities. Ten sticklebacks were analyzed. Two groups of 5 each guppies were tested: one group was fed ordinary commercial fish food; the other, chopped earthworms.

Results. The results are summarized in Table I, which shows that there is a mean value of 4.88% fatty acids, 0.32% cholesterol, and 1.87% phospholipids in the Arctic stickleback.

Table I also shows that the group of guppies fed on commercial fish food have the following mean lipid values: fatty acids, 8.87%; cholesterol, 0.56%; phospholipid, 2.31%. Guppies fed on chopped worms have lipid values practically identical with those fed commercial fish food.

Discussion. The results indicate that there is about the same amount of cholesterol in the guppies on either diet, but that there is less cholesterol in the Arctic fish than in the guppies. Total fatty acids are higher in the guppies, on either diet, than in the Arctic stickleback. The phospholipid content of all

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* Supported by a grant from the Arctic Institute of North America with funds provided by the Office of Naval Research. Sincere thanks are due Dr. Laurence Irving, director, and the staff of the Arctic Research Laboratory for invaluable aid rendered during the work in the field.

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TABLE I.
Results of Lipid Analyses of the Arctic Stickleback and of 2 Groups of Guppies.
All values expressed in percent fresh tissue.

Substance	Arctic stickleback	Guppies fed commercial fish food	Guppies fed earthworms
Fatty acid—mean	4.88	8.87	7.30
Range	2.46-7.06	8.14-9.94	6.22-9.33
Cholesterol—mean	0.32	0.56	0.55
Range	0.29-0.35	0.4-0.93	0.44-0.63
Lipid P—mean	0.08	0.09	0.08
Range	0.06-0.08	0.07-0.12	0.06-0.09
Phospholipid—mean	1.87	2.31	2.05
Range	1.57-2.08	1.83-2.60	1.52-2.31

TABLE II.
Table Showing the Various Lipid Ratios for the Arctic Stickleback and for the 2 Groups of Guppies. The ratios were calculated from the means given in previous table.

Group	Chol/fa	Chol/phosph	Chol/lipid P	Fa/lipid P
Stickleback	.07	.17	4.0	61.0
Guppy—worm fed	.08	.27	6.9	91.0
Fish food	.06	.24	6.2	99.5

3 groups is the same.

Calculations of the various lipid ratios were made for each group (Table II). Examination of these ratios indicates that, in relation to cholesterol, there is more phospholipid in the Arctic fish than in any of the guppies. It is maintained that the higher the ratio, cholesterol/lipid phosphorus, the greater the water content of the tissue in question.[6] If this generalization is universally true, the Arctic stickleback contains less water than does the guppy. It might be that this lower water content is correlated with the ability of the stickleback to withstand freezing.[7]

The fatty acid/lipid phosphorus ratios show that in the Arctic fish there is much less fatty acid, in proportion to phospholipid, than in the guppies. This may indicate that the turnover of fats is more rapid in the stickleback than in the guppies.

There is an interesting parallelism in the lipid content of the whole carcasses of guppies fed on commercial food and liver lipids in various fish. For example, Weill[8] found the liver cholesterol in various fish to be as follows (percent wet weight): *Truta fluvialitis,* 0.68; *Gadus merlangus,* 0.54; *Solea vulgaris,* 0.47. These values are quite close to that of 0.56% cholesterol in the guppies fed commercial food (Table I).

Mottram[9] reported that in the liver of the plaice fatty acids amount to 8.86% of the fresh tissue. This value compares closely with that of 8.87% fatty acids in whole guppies (Table I).

Similarly, Javillier and co-workers[10] found that in the liver of the carp lipid phosphorus amounts to 0.099% fresh tissue, a value similar to 0.09% for lipid phosphorus in the whole guppies. The Arctic stickleback also contains a similar amount of lipid phosphorus 0.08% (Table I). although its other lipids do not resemble so closely those of the guppy.

The present results indicate that in the stickleback phospholipids are high in proportion to other lipids. Such a condition suggests that in the single Arctic fish studied lipid metabolism is proceeding at a higher level than in the guppies, for phospholipid level is a good indication of overall level of fat metabolism.[1] One might expect just such a condition as an adaptation on the part of the stickleback to Arctic living: the high caloric values of the fats make them ideal sources of energy in an environment such as the Arctic.

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[6] Mayer, A., and Schaeffer, G., *J. physiol. path. gen.*, 1913, **15**, 771.

[7] Pouchet, F. A., *J. Anat. Physiol.*, 1866, **3**, 1.

[8] Weill, J., *C. R. Acad. Sci.*, 1914, **158**, 642.

[9] Mottram, V. H., *J. Physiol.*, 1912, **45**, 363.

[10] Javillier, M., Cremieu, A., and Hinglais, A., *Bull. Soc. chim. biol.*, 1928, **10**, 327.

Other adaptations of a metabolic nature are known to exist in Arctic fish: polar cod tissue shows Q_{02}-temperature curves peculiarly adapted to functioning at low temperatures.[11]

Summary. 1. Whole Arctic sticklebacks and ordinary guppies were analyzed for fatty acids, cholesterol and lipid phosphorus.

2. It was found that diet does not have an appreciable effect on the lipids in the guppies.

3. Cholesterol and fatty acid are higher in the guppies than in the Arctic fish. On the other hand phospholipid is essentially the same in all the fish examined.

4. There is more phospholipid in relation to cholesterol in the Arctic sticklebacks than in the guppies, a fact which may be interpreted as indicating a higher level of fat turnover in the former than in the latter.

5. The ratio of cholesterol/lipid phosphorus is lower in the stickleback than in the guppy, indicating a lower water content in the tissues of the former. This may be correlated with the reported ability of the stickleback to resist freezing injuries.

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[11] Field, J., and Peiss, C. N., *Fed. Proc.*, 1949, **8**, 44.

Received October 20, 1949. P.S.E.B.M., 1949, **72**.

Electroencephalographic Changes Associated with Painful and Non-Painful Peripheral Stimulation. (17454)

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This work was undertaken to ascertain whether the perception of pain is associated with a specific change in the electroencephalogram (E.E.G.), which might be used as an objective criterion of pain.

Methods. A Grass Electoencephalograph was used with 6 or 8 electrodes in 17 experiments on as many subjects. In addition to the usual 8 electrodes (2 frontals, 2 parietals, 2 temporals and 2 occipitals) the Grinker needle electrode[1] in the sphenoid bone was employed in 2 experiments, and a saline saturated cotton electrode pressed against the posterior pharyngeal wall in one. For the purpose of exploring any changes from the region of the base of the skull or hypothalamus near the foramen ovale in still another experiment an insulated needle electrode was inserted (under infiltration anesthesia) between the mandible and zygoma to a depth of 5.5 cm. Monopolar registration was employed with the lobe of the ear as indifferent electrode. Pain was produced in several ways in each experiment. (a) A Von Frey hair stimulator was applied to a previously determined pain point on the right forearm with a pressure of 8 g. (b) The web of the skin between the thumb and index finger of the right hand was pressed between a forceps. (c) As a control in two experiments heat, cold and touch were applied to the right forearm without causing pain.

Results. In all experiments a definite change in the E.E.G. occurred. It consisted of a decrease in amplitude, which was in the nature of a loss of electrical entropy and must be separated from the attention wave, which frequently appeared as an additional factor. The change was not specific for a pain stimulus or sensation since a similar response was obtained with heat, cold and touch. The decrease was bilateral and was not confined to any large cortical area, though it was more pronounced in the parietal, occipital and temporal region in a decreasing order of magnitude of change.

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[1] Grinker, R. R., and Serota, H. M., *J. Neurophysiol.*, 1938, **1**, 573.

Bipolar registration, shifting of electrodes, use of needle electrodes or coupling of 2 to 8 electrodes did not yield any essential improvement. The changes in the records from the base of the skull were similar to those observed from the cortical area.

In 4 experiments with morphine an additional decrease occurred to some extent on the application of the pain stimulus. Further tests were not made because pain and morphine[2] tend to produce both flattening of the middle range of waves which makes reliable interpretation of experimental data practically impossible.

Summary. The possibility of E.E.G. registration of pain was examined in 17 human subjects. A decrease in amplitude of the waves was observed, which was more evident in the parietal, occipital, temporal and frontal in a descending order. This change, however, is not dependent on the nature and degree of pain and it is not specific as it occurred with cold, heat and touch as well.

We desire to acknowledge the assistance of Dr. F. A. Gibbs and Mrs. E. L. Gibbs in certain aspects of this study.

Received July 27, 1949. P.S.E.B.M., 1949, **72**.

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[2] Gibbs, F. A., and Maltby, G. L., *J. Pharm. and Exp. Therap.*, 1943, **78**, 1.

Experimental Pulmonary Edema. II. Pathogenesis of Pulmonary Edema Caused by Ammonium Ion.* (17455)

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Koenig and Koenig[1] have described an acute pulmonary edema following the administration of ammonium salts to different species. It appears to be specifically a result of the ammonium ion. The occurrence and gross appearance of this edema resembles epinephrine induced pulmonary edema and in an effort to obtain information about the mechanism of the ammonium pathology a study has been made of its relation to edema due to epinephrine and adrenergic stimuli.

Experimental. Epinephrine induced pulmonary edema may be prevented by adrenergic blocking agents.[2,3] Their influence was observed upon ammonium pulmonary edema. Unfasted guinea pigs were divided into groups with the same sex distribution. All groups were lightly etherized to pass a stomach tube. Group 1, the controls, were given a sham dose of 10 cc water per kg body weight. All other groups received 1200 mg/kg of a 12% solution of NH_4Cl by oral gavage. Ten minutes before receiving the ammonium salt groups 3, 4 and 5 respectively received by intravenous injection in 1 cc saline/kg; 2 mg/kg of a B-haloethylamine, N-(9-fluorenyl)-N-ethyl-B-chloroethylamine-HCl ("SKF-501"); 2.5 mg/kg N-(2-chloroethyl)-N-ethylbenzhydrylamine-HCl ("SY-2"), which like "SKF-501" is an effective adrenergic blocking agent; and 1.5 mg/kg of the potent antihistaminic N-B-dimethylaminopropylthiodiphenylamine - HCl ("Phenergan", 3277 R.P.). The presence of pulmonary edema was determined at an autopsy by gross inspection and lung weight was used as a measure of its extent.

The summarized data comprise Table I. Group 1 was killed with ether while all of the animals in the other groups succumbed with convulsions 5-30 minutes after receiving the ammonium salt. The two adrenergic blocking agents and the antihistaminic all were

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* This study was supported by a grant from the Life Insurance Medical Research Fund.

[1] Koenig, H., and Koenig, R., Proc. Soc. Exp. Biol. and Med., 1949, **70**, 375.

[2] Stone, C. A., and Loew, E. R., Proc. Soc. Exp. Biol. and Med., 1949, **71**, 122.

[3] MacKay, E. M., and Pecka, E. F., Jr., Proc. Soc. Exp. Biol. and Med., 1949, **71**, 669.

TABLE I.
Prevention of Ammonium Chloride Pulmonary Edema in Guinea Pigs with Adrenergic Blocking Agents.

Group	No. of animals In group	No. of animals With edema	Avg body wt, g	Lung wt, g/100 g body wt
		Controls		
1	18	0	736	0.69 ± .11
		1200 mg NH_4Cl/kg		
2	24	24	680	1.50 ± .21
1200 mg NH_4Cl/kg preceded by 2 mg/kg of N-(9-fluorenyl)-N-ethyl-B-chlorethylamine*				
3	18	0	645	0.71 ± .11
1200 mg NH_4Cl/kg preceded by 2.5 mg/kg N-(2-chloroethyl)-N-ethylbenzhydrylamine • HCl†				
4	12	0	685	0.65 ± .15
1200 mg NH_4Cl/kg preceded by 1.5 mg/kg N-B-dimethylaminopropylthiodiphenylamine • HCl‡				
5	12	11	676	1.40 ± .25

* We are indebted to Dr. Glenn E. Ullyot, of Smith, Kline and French in Philadelphia for a supply of this compound (SKF-501).

† Dr. Leon A. Sweet of Parke, Davis and Co., Detroit, Mich., sent this material (SY-2) to us.

‡ (Phenergan, 3277 R.P.) supplied through the courtesy of Dr. A. Gibson, Assoc. Medical Director, Merck and Co., Rahway, N. J.

without influence upon mortality. Since both of the adrenergic blocking agents completely prevented the ammonium edema but not the mortality, we may conclude that pulmonary edema need not be the cause of death from toxic doses of ammonium salts. The anthistaminic was without effect on the ammonium edema, which in this respect resembles the fulminating pulmonary edema due to epinephrine.[2]

Epinephrine-induced pulmonary edema appears to be due to increased pulmonary pressure which it produces[2] possibly not directly, even though the vascular tree of the lungs is richly supplied with sympathetic fibres, but as a result of the high systemic arterial pressures. The complete protection against epinephrine edema afforded by adrenergic blocking drugs[2,3] which pharmacologically block excitatory actions of epinephrine such as vasoconstriction, is also true of the ammonium edema which suggests that the latter might also result from adrenergic stimuli. Some years ago we suggested[4] that the pulmonary edema of experimental hypoglycemia might be due to the release of epinephrine by the adrenal medulla. If this were true, medullectomy of the adrenal in the rat, where this procedure is feasible, could affect the development of the ammonium edema. We tried to test this but were unable to produce this type of pulmonary edema in our rats (Slonaker Strain[5]). Groups of 6 rats each had lung weights of 0.53 ± .06 g/100 g body weight (controls), 0.55 ± .07 g/100 g body weight (normals receiving 0.4 cc 10% NH_4Cl/100 g body weight intraperitoneally) and 0.48 ± .09 g/100 g body weight (demedullectomized rats receiving the same dose of NH_4Cl), respectively. This was equivalent to the dose of ammonium salt used by the Koenigs to produce edema in their rats. Nor could we produce pulmonary edema in the rat with ammonium salts whatever the dose when given *per os* as well as intraperitoneally. This is additional evidence that mortality from ammonium salts is not necessarily a result of the pulmonary edema which they frequently cause, since our rats all died. The same thing was true in albino mice of an unknown strain. We also are in disagreement with the Koenigs as to the action of ammonium salts in the rabbit. They found no edema. We regularly found lung edema after the administration of am-

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[4] Sherrill, J. W., and MacKay, E. M., *Arch. Int. Med.*, 1939, **64**, 907.

[5] MacKay, L. L., and MacKay, E. M., *Am. J. Physiol.*, 1927, **83**, 179.

TABLE II.
Influence of Ammonium Chloride on Blood Sugar.

			Average blood sugar concentration—mg%*	
Time in min.	1 Controls	2 NH_4Cl	3† NH_4Cl preceded by ''SKF-501''	4‡ NH_4Cl preceded by tetraethylammonium
0	112	132	140	136
10	116	216	224	206
17	118		208	
20			260	220
22		272	256	244
24	120	296	272	282

* 3 unfasted guinea pigs in each group.
† Doses of NH_4Cl and ''SKF-501'' as in Table I.
‡ Dose of tetraethylammonium bromide 10 mg/kg subcutaneously.

monium chloride and it was prevented by the adrenergic blocking agents. The control lungs always appeared much more edematous than their weight would indicate. We have often noted a disproportion between the extent of edema and the amount of fluid which collected in the alveoli and bronchioles. Groups of four 2 kg rabbits were given 1.25 g/kg of 25% NH_4Cl by stomach tube. Two groups were given the blocking agents in the same dose used in the guinea pig experiments. The lungs of Group 1 (controls) averaged 0.46 g, Group 2 (NH_4Cl treated) 0.71 g, Group 3 (NH_4Cl preceded by "SKF-501", see Group 3, Table I) 0.45 g and Group 4 (NH_4Cl preceded by "SY-2", see Group 4, Table I) 0.42 g/100 g body weight, respectively. There was no edema except in the lungs of Group 2.

Ammonium salts might exert an adrenergic effect by direct action on sympathetic nerve endings, or by direct or reflex central stimulation. Ammonia has long been known as a most effective means of stimulating the brain medulla reflexly[6] and in addition to lung edema, the Koenigs have found pathology resulting from fatal doses of ammonium salts. Hypnotics have been claimed to prevent lung edema due to epinephrine.[7] Ethyl ether produces an anesthesia characterized by complete paralysis of the motor reflex centers in the cord and some depression of the medullary centers. When our animals were continued under a deep ether anesthesia in place of stopping the anesthetic after the light etherization used for gavage to give the ammonium chloride, they lived as long as similar unanesthetized controls and died without the usual convulsions typical of toxic doses of ammonium salts. Pulmonary edema was always absent. The average lung weight of 4 etherized ammonium chloride pigs was 0.57 in comparison with 1.34 g/100 g body weight for a similar group of non-etherized NH_4Cl controls. We interpret this as evidence in favor of the ammonium-induced pulmonary edema being a result of reflex or direct stimulation of the medullary centers which results in an outflow of excessive adrenergic stimuli. The stimuli do not necessarily act on the pulmonary circulation primarily but as in epinephrine pulmonary edema[3] may produce left heart failure with an increased pulmonary venous pressure and the inevitable edema.

The administration of ammonium chloride causes a marked hyperglycemia which might be interpreted as due to a release of epinephrine by the adrenal medulla. However ammonium salts have been reported[8,9] to have no effect on adrenal release of epinephrine and they may induce a hyperglycemia in adrenalectomized rabbits.[10] We have found (Table II) that neither an adrenergic blocking agent nor tetraethlammonium which blocks the release of epinephrine by the adrenal medulla[11] influences the hyperglycemia due to ammonium salts in guinea pigs and conclude that the carbohydrate disturbance is not directly related to the mechanism of the pulmonary edema.

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[6] Sollman, T., A Manual of Pharmacology, 3rd ed., p. 859, W. B. Saunders Co., Philadelphia, 1928.

[7] Luisada, A., *Arch. Exp. Path.*, 1928, **132**, 313.

[8] Houssay, B. A., and Molinelli, E. A., *Compt. rend. Soc. biol.*, 1925, **93**, 1156.

[9] Shoji, J., *Tohoku J. Exp. Med.*, 1937, **30**, 259.

[10] Satake, Y., *Tohoku J. Exp. Med.*, 1926, **8**, 26.

Summary 1. The acute pulmonary edema which follows the administration of ammonium salts to guinea pigs may be entirely prevented by prior administration of the adrenergic blocking agents, N-(9-fluorenyl)-N-ethyl-B-chloroethylamine-HCl ("SKF-501") and N-(2-chloroethyl)-N-ethyl-benzhydrylamine-HCl ("SY-2"). Less marked lung edema is produced in rabbits by ammonium chloride and it is also prevented by the blocking agents.

2. A potent antihistaminic agent, N-B-dimethylamino-propylthiodiphenylamine- HCl ("Phenergan-3277 R.P."), was without effect on the incidence of ammonium pulmonary edema, which in this respect as well as the manner it is influenced by adrenergic blocking agents, resembles epinephrine-induced pulmonary edema.

3. A central nervous system depressant in the form of continuous ether anesthesia prevents the occurrence of ammonium pulmonary edema. Along with the effect of adrenergic blocking agents this is construed as evidence for the mechanism of this edema being due to adrenergic stimuli of reflex or direct central stimulation origin.

4. Ammonium salts cause a striking hyperglycemia. Since adrenergic blocking agents of the type used as well as tetraethylammonium which blocks epinephrine release by the adrenal medulla fail to prevent the hyperglycemia response it is believed that ammonium hyperglycemia is not directly related to the edema formation.

5. Although pulmonary edema sometimes may be the cause of death from toxic doses of ammonium salts, this is not true in the Slonaker strain of rats or our strain of albino mice both of which fail to develop edema. Nor is this the cause of death in guinea pigs or rabbits when an adrenergic blocking agent is used to prevent the edema, as the animals succumb as a result of other effects of the ammonium ion.

Addendum: When this manuscript was ready for submission to the Editors, an article by the Koenigs (Koenig, H., and Koenig, R., *Am. J. Physiol.*, 1949, **158**, 1) on the pathogenesis of ammonium pulmonary edema appeared. Insofar as our observations are similar the conclusions are parallel.

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[11] Morrison, J. L., and Farrar, C. H., PROC. SOC. EXP. BIOL. AND MED., 1949, **71**, 235.

Received Sept. 30, 1949. P.S.E.B.M., 1949, **72**.

Effect of Adrenal Cortical Extract and of X-Radiation on Serum Proteins of Mice. (17456)

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It has been reported previously from this laboratory that chronic injections of pituitary adrenotrophic hormone in mice produced an increase in the total serum proteins.[1] Moreover, a single injection of adrenotrophin or of adrenal cortical steroids in oil in rabbits was observed to cause an elevation of circulating β- and γ-globulins within a few hours after hormone administration.[2] Electrophoretic analysis also revealed an increase in these serum globulins in normal mice exposed to a single dose of X-rays.[3] It was suggested that the influence of radiation on blood globulin levels in intact mice was chiefly a result of the direct involution of lymphoid tissue by large radiation doses. An augmentation of pituitary-adrenal cortical controlled lymphocytolysis, with accompanying blood globulin elevation, was also demonstrated to occur as a result of the response of the endocrine mechanism to the action of radiation as a non-specific stimulus.

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† Present address: School of Medicine, University of California at Los Angeles, Los Angeles, Calif.

[1] White, A., and Dougherty, T. F., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 26.

[2] White, A., and Dougherty, T. F., *Endocrinology*, 1945, **36**, 207.

A review of the literature[4] indicates that conflicting data have been obtained in efforts to establish definitely a relationship between pituitary-adrenal cortical secretion and serum protein levels. Simonnet[5] injected adrenotrophic hormone or adrenal cortical extracts into rats of both sexes and of varying ages, and found a rise in total blood globulin occurring most consistently in the 2-month-old male rat. On the other hand, Li and Reinhardt[6] were unable to alter the electrophoretic serum protein pattern of normal or of hypophysectomized rats by giving single or chronic injections of adrenotrophic hormone. It may be noted that the data of these investigators show an approximately 100% difference in values for γ-globulin concentrations in 2 groups of control rats.

In rats and dogs exposed to physical and chemical stimuli which augment pituitary-adrenal cortical secretion, Chanutin and his associates[7,8] found rather uniformly an increase in the serum α-globulin fraction and the appearance of boundary anomalies in the β-globulin component. Arocha and De Venanzi[9] reported briefly that serum globulin increased in normal dogs following intravenous injections of epinephrine, and Mondolfo and De-Lerner[10] confirmed the observations of Chase, White, and Dougherty[11] that adrenal cortical extract injections, accompanying antigen administration, resulted in a greater rate and degree of elevation of blood immune globulin. However, Eisen and co-workers[12] found identical concentrations of serum antibodies and γ-globulin in adrenalectomized rats injected repeatedly during immunization with adrenal cortical extract, and in operated animals not receiving hormone.

Pituitary adrenotrophic hormone or adrenal cortical extract injections have failed generally to influence the level of serum globulin in human subjects. This has been the experience of Kelley and Adams,[13] Forsham and co-workers,[14] Mason and co-workers[15] and Frieden and White,[16] following administration of adrenotrophic hormone or adrenal cortical extracts. In addition, exposure of patients to X-radiation, nitrogen mustard therapy, or electric shock treatment was rather uniformly without influence on the electrophoretic pattern of the serum proteins, although in two instances significant rises were seen in the serum γ-globulin fraction after x-radiation.[16]

In view of the nature of the conflicting evidence in a variety of species, experiments have been initiated to investigate systematically the possible variables which may be involved in influencing the nature and extent of the response of the serum protein pattern to circumstances of augmented levels of circulating adrenal cortical steroid hormones. The present investigation deals with the response in mice. The previously reported[3] electrophoretic experiments with serum obtained from mice exposed to X-radiation were conducted in phosphate buffer at pH 7.8. Under these circumstances, the separation of the γ-globulin component from the salt boundary is unsatisfactory, and errors may arise in evaluating correctly the contribution of the γ-globulin component to the total area in this region of the electrophoretic pattern. In addition, when this phosphate buffer is employed, a satisfactory separation of the α-globulin fraction into its two commonly recognized individual components is not obtained. Another possible source of error in electrophoretic studies of sera results from the fact that the precise concentration of the β-globulin component may be somewhat obscured by the presence of lipid in this protein fraction, resulting in an apparent, but not true, increase in β-globulin concentration. This factor would seem to be of particular significance in circumstances of augmented pituitary-adrenal cortical secretion, particularly since the adrenal cortical steroids produce a mobilization of lipid to the liver.

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[3] Dougherty, T. F., and White, A., *Endocrinology*, 1946, **39**, 370.

[4] White, A., *Ann. Rev. Physiol.*, 1949, **11**, 355.

[5] Simonnet, H., *Bull. Acad. Med.* (Paris), 1947, **131**, 245.

[6] Li, C. H., and Reinhardt, W. O., *J. Biol. Chem.*, 1947, **167**, 487.

[7] Chanutin, A., and Gjessing, E. C., *J. Biol. Chem.*, 1946, **165**, 421.

[8] Gjessing, E. C., Ludewig, S., and Chanutin, A., *J. Biol. Chem.*, 1947, **170**, 551.

[9] Arocha, H. G., and De Venanzi, F., *Proc. Intern. Physiol. Congr.*, 1949, XVII Congress, 129.

[10] Mondolfo, H., and De Lerner, S. J., *Farmalecta*, 1947, **2**, 171.

[11] Chase, J. H., White, A., and Dougherty, T. F., *J. Immunol.*, 1946, **52**, 101.

[12] Eisen, H. N., Mayer, M. M., Moore, D. H., Tarr, R. R., and Stoerk, H. C., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 301.

[13] Kelley, V. C., and Adams, J. M., *J. Pediat.*, 1948, **32**, 282.

[14] Forsham, P. H., Thorn, G. W., Prunty, F. T. G., and Hills, A. G., *J. Clin. Endocrinology*, 1948, **8**, 15.

[15] Mason, H. L., Power, M. H., Rynearson, E. H., Ciaramelli, L. C., Li, C. H., and Evans, H. M., *J. Clin. Endocrinology*, 1948, **8**, 1.

[16] Frieden, J., and White, A., unpublished results.

The present study has involved the electrophoretic examination of the serum proteins of normal mice, and of mice bearing a transplantable lymphosarcoma, both prior to and following either the injection of adrenal cortical extract, or exposure to a single, relatively large dose of X-radiation. Under the experimental conditions employed, no significant alterations were seen in the serum protein patterns as a consequence of exposure of the animals to either the hormonal or radiation treatment.

Methods. Mice of both sexes of the CBA (Strong) strain, 8 to 13 weeks of age, were used in all experiments. The animals were maintained on a diet of Purina Fox Chow supplemented with calf meal; food and water were available at all times. The tumor employed was obtained from Professor W. U. Gardner of the Department of Anatomy at Yale University. This tumor arose in an estrogen-treated mouse of the C_3H strain and has been transplanted for many successive generations. A transplant of the tumor takes in 100% of CBA mice. The transplanted tumor grows rapidly and kills the host in approximately 3 weeks. The tumor has been described in detail[17] and is composed of large cells which resemble reticular cells. Tumor transplants were made by implanting small pieces of the tumor subcutaneously. Since the mice with this tumor transplant live generally a maximum of three weeks, the tumor-bearing animals were used 2 weeks after the transplant had been made, at a time when a clearly palpable tumor was present.

Two preparations of adrenal cortical extract were used, one an aqueous adrenal cortical extract (Wilson) and the other a preparation of adrenal cortical steroids in oil (Upjohn). The animals subjected to X-rays received total body radiation. This was accomplished by placing 4 to 6 mice in a light cardboard box, which because of its shallowness prevented the mice from shielding one another. Radiation of all animals was delivered at the rate of 43 r per minute by using the following conditions: 125 Kv., 5 ma.; 30 cm skin target distance; 3 mm aluminum filter.[‡]

In order to obtain blood samples, each mouse was pithed, and the heart exposed and blood obtained by direct heart puncture, using a finely drawn glass pipette. Blood was pooled from groups of animals varying in number from 15 to 32, in order to obtain adequate serum for each electrophoretic experiment. The total serum protein and non-protein nitrogen contents of the pooled serum were determined by the micro-Kjeldahl technic. The serum was then diluted with 3 parts of 0.1 M sodium barbital buffer, pH 8.6, and dialyzed against the buffer for 2 or 3 days in the ice box. Electrophoretic runs were conducted in the Tiselius apparatus at 2°C for a period of approximately 3 to 4 hours, at which time satisfactory separation of the individual serum protein components had occurred. The concentrations of the various protein fractions were estimated from the descending diagrams

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[17] Gardner, W. U., Dougherty, T. F., and Williams, W. L., *Cancer Research*, 1944, **4**, 73.

[‡] Grateful acknowledgement is made to Dr. H. S. Kaplan for assistance in radiation of the animals.

TABLE I.
Electrophoretic Analyses of Mouse Sera.

Electrophoresis exp. No.	Animal group type	No. of mice for pooled sera	Total protein, g/%	Albumin,		Globulins									
						α_1		α_2		β		γ		$\beta + \gamma$	
				%	g	%	g	%	g	%	g	%	g	%	g
1	Normal, ♂	32	4.53	59.70	2.70	15.70	.71	9.45	.43	9.45	.43	3.48	.16	12.95	.59
2	”	23	4.82	63.43	3.05	16.50	.79	6.74	.32	9.75	.47	3.61	.17	13.76	.64
3	Normal, ♀	15	4.75	67.60	3.21	11.63	.55	6.71	.32	10.62	.51	3.35	.16	13.97	.67
4	Tumor, ♂	15	3.57	61.36	2.19	18.80	.67	5.45	.19	10.90	.39	2.48	.09	13.38	.48
5	” ♀	15	3.41	63.44	2.16	14.75	.50	7.05	.24	11.53	.39	3.21	.11	14.74	.50
6	Normal, ♂ and ♀[1]	16	6.08	63.05	3.84	18.22	1.11	5.61	.34	10.75	.65	2.39	.15	13.14	.80
7	Tumor, ♂ + ♀[2]	16	5.18	61.98	3.22	16.81	.87	5.43	.28	13.05	.68	2.73	.14	15.78	.82
8	” ♂ + ♀[3]	18	5.22	65.80	3.43	15.42	.81	3.72	.19	11.54	.60	3.72	.19	15.26	.79
9	Normal, ♂ + ♀[4]	18	4.96	66.20	3.28	15.10	.75	4.04	.20	11.60	.57	3.03	.15	14.63	.72
10	” ♂ + ♀[4]	18	5.86	68.40	4.01	12.55	.73	5.20	.31	10.82	.63	3.03	.18	13.85	.81

[1] 6 hr after 0.2 cc adrenal cortical steroids (oil), subcut.
[2] 3 hr after 0.5 cc ” ” ” ” ”
[3] 3 hr after 1.0 cc aqueous adrenal cortical extr., subcut.
[4] 6 hr after X-radiation (200 r).

of the electrophoretic patterns.

Results and discussion. Table I presents the results of the various analyses under the experimental conditions indicated in the table. It will be seen from the data that a single injection of adrenal cortical extract, or the exposure of mice to 200 r produced an increase in the total serum protein concentration, as compared to appropriate controls, in all experiments except that designated as No. 9. This increase is in agreement with previously published data.[1] However, it will also be seen from the table that no significant alteration occurred in the percentage distribution of the various serum protein components as determined electrophoretically. The slight alterations which were evident in several of the experiments, *e.g.*, No. 7, occurred in the β-globulin fraction. In view of the influence of altered lipid concentrations on the value for this protein component, it is unlikely that this increase is indicative of an elevated concentration of β-globulin.

It is unknown at the present time whether the relatively low initial level of total serum proteins in the control animals was a factor in determining possible responsiveness to the experimental conditions employed. In all experimental groups, there was a striking elevation in total serum proteins. It is possible that in a relatively protein-depleted animal, there is a generalized stimulus to protein production following hormone administration or exposure to X-radiation. However, in the experimental animals, there was no significant alteration in the relative distribution of the individual serum components.

The precise relation of pituitary-adrenal cortical secretion to the normal pattern of serum proteins would appear to wait further investigation. The conflicting data in the literature, taken together with the present experiments, suggest that variables exist which remain for further careful evaluation. These comments relate not only to the pattern of normal serum globulins, but also to immune globulins, about which there also have appeared conflicting data.

It may be pointed out that serum protein levels alone do not provide a complete evaluation of the possible relation of endocrine secretions to blood protein production and utilization. Thus, it has been demonstrated recently[18] that although the antibody level in the *blood* of immunized, normal and adrenalectomized rats may be at similar levels, the *tissues* of the adrenalectomized rats are producing antibody at a rate which is distinctly less than that of the unoperated animals. This suggests that the adrenalectomized animals are also removing antibody from the circulation at a rate which is less than normal. Thus, although the blood titer is similar in the two groups of animals, the presence of the adrenal does appear to influence the rate at which the antibody globulin enters and leaves the circulation. It may also be of significance that Mondolfo and DeLerner[10] reported recently that whereas relatively small doses of adrenal cortical extract may augment the rate of antibody production, significantly larger amounts of the same extract produced a suppression of antibody production as compared to the rate seen in animals receiving antigen alone.

Summary. Normal mice, or mice bearing a transplantable lymphosarcoma, were given single injections of adrenal cortical extract or exposed to a single radiation dose of 200 r (total body radiation). Examination of the serum protein patterns of these mice at 3 and at 6 hours after hormone injection, or at 6 hours after exposure to X-rays, revealed a striking increase in total serum protein levels. However, electrophoretic examination did not reveal significant alterations in the relative distribution of the serum proteins.

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[18] Roberts, S., and White, A., unpublished results.

Received October 17, 1949. P.S.E.B.M., 1949. **72**.

Cinchophen Choleresis and Production of Peptic Ulcer in Various Species.

(17457)

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Brugsch and Horsters[1] were the first to show that cinchophen, in both dogs and man caused an increase in the bile flow. Stransky[2] carried this study to rabbits and was unable to detect any choleretic effect of cinchophen in this species. Berman and Ivy[3] confirmed this finding and found in addition that the excretion of cinchophen in the bile of rabbits was very small.

Churchill and Van Wagoner[4] demonstrated that dogs receiving 20 to 30 times the human dose of cinchophen developed gastric or duodenal ulcers. This finding has since been confirmed very many times.[5–7] Shoji[8] found that rabbits were very resistant to the ulcerogenic action of this drug. Okada[9] likewise failed to produce cinchophen ulcers in either rabbits or guinea pigs. Schwarz and Simmonds[10] were unable to induce cinchophen gastric, or duodenal ulceration in rabbits or guinea pigs, but found cats very susceptible. The ability of cinchophen to cause liver damage in rats[11] has been noticed but there has been no report of its effect upon the gastrointestinal tract.

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[1] Brugsch, W., and Horsters, H., *Z. ges. Exp. Med.*, 1923, **38**, 367.

[2] Stransky, E., *Biochem. Z.*, 1925, **155**, 256.

[3] Berman, A. L., and Ivy, J. H., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 853.

[4] Churchill, T. P., and Van Wagoner, F. H., PROC. SOC. EXP. BIOL. AND MED., 1931, **28**, 581.

[5] Stalker, L. K., Bollman, J. L., and Mann, F. C., *Arch. Surg.*, 1937, **34**, 1172.

[6] Bollman, J. L., and Mann, F. C., *Proc. Staff Mayo Clinic*, 1935, **10**, 580.

[7] Nasio, J., *Rev. Gastroenterol.*, 1946, **13**, 195.

[8] Shoji, A., *Trans. Soc. Path. Jap.*, 1933, **23**, 520.

[9] Okada, S., *Trans. Soc. Path. Jap.*, 1933, **23**, 522.

[10] Schwarz, S. O., and Simmonds, J. P., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1133.

[11] Barbour, H. G., and Fisk, M. E., *J. Pharm. and Exp. Therap.*, 1933, **48**, 341.

TABLE I.
Effect of Cinchophen Upon Bile Output in Various Animals and Its Excretion in the Bile.

No. of animals	Species	Avg wt, g	Cinchophen administered, mg/kg	Bile output				% recovery of cinchophen in bile in 1st 2 hr
				Control, ml in 30 min.	Control, ml/kg hr	1st 30 min. after cinchophen	Increase, %	
4	Rabbits	2,400	100	4.5	3.74	4.25	0	1.75
13	Rats	241	100	0.266	2.2	0.239	0	5.1
14	Guinea pigs	569	100	2.2	7.2	3.0	36.3*	27.7
11	Cats	2,850	100	0.77	0.54	0.95	23	2.18

* This figure only is statistically significant ($P = <0.01$).

Except in the case of the rabbit and dog, it has not previously been shown whether or not any relationship exists between the susceptibility of an animal to cinchophen peptic ulcer and the animal's choleretic response to this drug.

Methods. Two series of experiments were conducted, acute experiments to determine the effect of cinchophen upon the bile flow and chronic feeding experiments to determine the susceptibility of the various animals to cinchophen ulceration.

Acute experiments. Cats, rabbits, guinea pigs and rats were used in this study. Dogs were not used as they have been adequately studied by others.[1,12] The animals having been fasted for the preceding 12 hours were anesthetized with "Nembutal". The gallbladder, where present, was tied off and the common duct cannulated using plastic tubing. In rats, a glass cannula of near-capillary dimensions attached by rubber tubing to a horizontal graduated 1 ml pipette was used. In the other species the bile was collected in 10 ml graduated cylinders. The volume was measured half-hourly. After a steady control output had been reached, cinchophen dissolved in $\frac{N}{10}$ NaOH was administered intravenously. In those animals failing to respond, the reactability was tested with sodium dehydrocholate ("Decholin"). Animals failing to give a response to sodium dehydrocholate were discarded.

Chronic Feeding Experiments. In these studies the same four species were used with the addition of dogs. The latter were used to determine the ulcerogenic potency of the cinchophen. The drug was administered to cats, dogs, guinea pigs and rabbits daily in gelatin capsules. It was in addition administered subcutaneously to another series as an aqueous solution of the sodium salt to guinea pigs, rabbits and rats. To still another series of rabbits and rats a suspension of the free acid in tragacanth was administered by stomach tube. In these species this mode of administration was found more convenient than gelatin capsules. As it was observed that the animals most susceptible to cinchophen ulcer were carnivora, a series of rabbits, rats and guinea pigs was fed the same proprietary meat dog food as were the dogs and cats. In all cases control animals were fed the same diet without cinchophen as the test animals.

It is now well known that choleresis results from the administration of cinchophen both

TABLE II.
Apparent Relationship Between Cinchophen Excretion in Cats' Bile and Choleresis Induced by Cinchophen.

Cat. no.	% cinchophen recovery in bile in 1st 2 hr.	% increase in bile flow
1	0.13	0
2	1.13	0
3	1.7	16.75
4	2.5	100
5	5.65	117.5

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[12] Bradley, W. B., and Ivy, A. C., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 143.

TABLE III.
Incidence of Ulceration in Various Animals Resulting from Cinchophen Feeding.

No. of animals	Species	Dose range, mg/kg	Diet	Survival time, days	No. of animals showing	
					Ulcers	Erosions
21	Rabbits	100-600	Normal	12-32	0	0
11	’’	100-200	Meat	12	0	2
20	Guinea	100-230	Normal	16.5	1	0
10	pigs	300	’’	7.1	6	0
6	’’	400	’’	5	1	0
24	Rats	100-400	Meat and normal	S 42-56	0	0
11*	’’	500 and 1500	Normal	S 15	2	5
12	’’	600-700	’’	S 10	10	1
6	Cats	100	’’	9	3	1
4	’’	200	’’	6	3	0
1	Dogs	100	’’	S 62	0	0
6	’’	200	’’	14	6	0

* These animals were fed 500 mg/kg for 13 days and given 1,500 mg/kg on the 14th, and killed on the 15th.

S = Sacrificed.

by vein and orally to acute and chronic biliary fistula dogs.[12]

Results. It will be seen from Table I that guinea pigs show both the greatest choleretic response to cinchophen and also the greatest secretion of cinchophen in the bile. Rats and rabbits show no choleretic response. The rats, however, secreted appreciable quantities of cinchophen in the bile whereas the rabbits as was previously shown by Berman and Ivy[3] did not. Our results in cats were not clear cut. The average figures show a slight increase, but this was not statistically significant, many of the animals failed to respond to sodium dehydrocholate and were therefore not included in the results. It will be seen from Table II that the cinchophen output in the cats' bile shows individual variation, but nevertheless the magnitude of the choleretic effect appears to vary directly as the cinchophen output.

It was found (Table III) that among the species studied rabbits alone failed to develop cinchophen ulcers. Two gastric erosions occurred amongst the meat fed animals, but as these appeared with equal frequency in the controls fed meat without cinchophen the causative factor was thought to be the presence of fragments of bone in the meat.

The only ulceration seen in rats occurred in a series fed 500 mg/kg cinchophen daily for 13 days. On the 14th day, 1,500 mg/kg was administered. Two ulcers and a number of erosions (Table III) were observed at autopsy on the 15th day, and in another series in which 10 of one dozen rats, fed 600-700 mg/kg developed ulcers within 10 days (Fig. 1). The stomachs of all were full of food thus excluding starvation as a possible cause of the gastric lesions. All the ulcers seen occurred in the glandular part of the stomach. The meat diet seemed to be without effect.

Ulcers occurred in guinea pigs more frequently than in rats, but still with less frequency than in cats or dogs. They occurred only when doses in the neighborhood of 300 mg/kg were given daily. Above and below this dose level cinchophen killed the animals but ulcers were not seen. Meat feeding to guinea pigs over long periods of time was found impossible as the animals would starve rather than eat the diet. In our earlier investigation,[13] doses of 200 mg/kg were used and ulcers were not produced. A group of control animals fasted for one week without being given cinchophen developed the charac-

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[13] Magee, D. F., Kim, K. S., and Ivy, A. C., *Fed. Proc.*, 1949, **8**, 103.

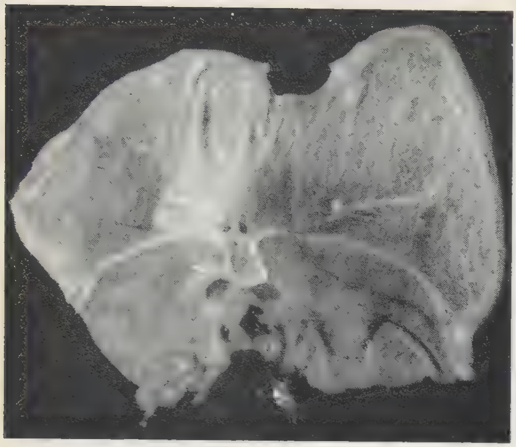

FIG. 1.
Ulcers produced in the rat's stomach by feeding 600 mg/kg cinchophen for 10 days.

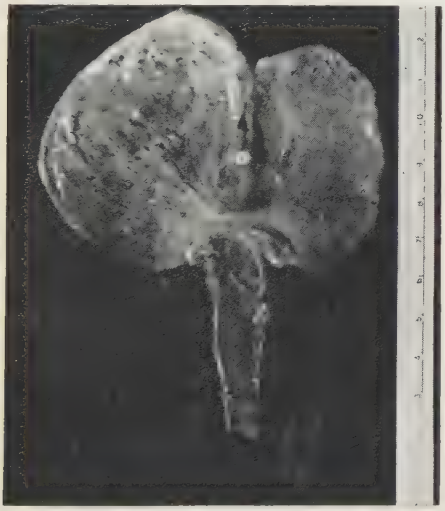

FIG. 2.
Typical gastric ulceration resulting from feeding 300 mg/kg cinchophen to guinea pigs.

teristic mottled yellow liver, seen in all animals except dogs, but no ulcers appeared. This excludes the possibility that cinchophen produced ulceration is secondary to loss of appetite and subsequent starvation.

The ulcers seen in guinea pigs were quite unlike those seen in other animals. They were almost always multiple and were confined almost exclusively to the fundic part of the stomach (Fig. 2) whereas in cats and dogs, in both of which ulcers occurred easily, they were pyloric, usually single, occasionally 3 or 4 were observed in cats. In guinea pigs there were seldom less than 20.

Similar ulcers were produced in guinea pigs using sodium dehydrocholate in doses of 200 mg/kg/day orally or subcutaneously often within 24 hours. Sellards[14] and Tsuruta[15] have produced this characteristic guinea pig type of ulceration with sodium glycocholate and the sodium salts of conjugated cholic acids obtained from ox bile.

Discussion. It appears, at least superficially, that cinchophen ulceration can be produced most easily in those species which show a choleresis, but the choleretic response in cats to cinchophen is so small and variable as to be statistically insignificant, and moreover it is not certain that the ulcers in the guinea pig are of the same origin as those occurring in cats and dogs. Sodium dehydrocholate administered to guinea pigs will produce ulcers almost identical in site and appearance with those resulting from cinchophen. This has not been seen in the other animals. The ulcers produced in rats were in response to such enormous doses of cinchophen that a direct effect upon the mucosa cannot be excluded.

No correlation appears to exist between the cinchophen output in the bile and the species susceptibility to cinchophen ulceration. Rats and especially guinea pigs secrete very much more cinchophen in their bile than do cats and yet cats were the most ulcer susceptible animals studied.

The only factor which is common to all ulcer resisting species is that they have a high control output of bile. However, the most susceptible of the resisting species, the guinea pig, has the highest basal bile flow (Table I). Cats and dogs, both of which secrete approximatey 0.5 ml per kg/hr are very susceptible. It is well known that dogs totally deprived of bile often develop spontaneous ulcers. A possibility which must be considered is that cinchophen has a different metabolic pathway in the more resistant group of animals from that in the less resistant species.

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[14] Sellards, A. W., *Arch. Int. Med.*, 1909, **4**, 502.

[15] Tsuruta, T., *Cincinnati Med. Bull.*, 1931, **6**, 110.

Summary and conclusions. A choleretic response to cinchophen was observed in guinea pigs.

The cinchophen output in the bile was found to be greatest in guinea pigs and to decrease in the following order: rats, cats and rabbits.

The following is the order found for susceptibility to cinchophen ulceration: cat, dog, guinea pig and rat. Rabbits were found completely resistant.

There appears to be no obvious relationship between the species susceptibility to cinchophen ulceration and the choleretic response of that species to cinchophen or to the concentration of cinchophen in the bile after intravenous administration.

It would appear that the ulcer susceptible animals investigated have a low basal bile flow whilst the unsusceptible have a high flow.

Received October 4, 1949. P.S.E.B.M., 1949, **72**.

Focal Neurological Lesions Produced by Microwave Irradiation. (17458)

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Prewar and wartime research allowed the development of electronic equipment capable of efficiently producing large quantities of radiation above 1000 megacycles (microwaves). Notable among these advances was the development of the magnetron tube which is used in radar equipment[1] and has been incorporated recently in a microwave diathermy machine.* The frequency at which this machine operates (2450 megacycles, 12.2 cm) allows good penetration of animal tissue and even of bone, as demonstrated by Osborne and Fredericks[2] and Krusen, Herrick and Wakim.[3] These findings and the fact that radiation at this frequency travels in substantially straight lines suggests that the application of toxic doses of this radiation to a small area of the cerebral cortex is possible without injury to the scalp and skull.

However, a review of the literature reveals no report of the utilization of microwave radiation for the artificial production of focal neurological lesions. With the technic to be described focal lesions were produced in rabbit brains without surgical procedure. The site of the cortical lesion, moreover, was accurately determined before the irradiation.

Method. Adult rabbits weighing about 3 kg were utilized. Under intravenous pentothal anesthesia sufficient to immobilize the animal (80-100 mg) the animal's head was placed on a wooden block approximately 7 cm in thickness. The area of the scalp over the proposed site of irradiation was shaved. A thin copper shield with an oval hole 2 x 3 cm was then placed with the long axis of the hole parallel to the rabbit and over the proposed site, in the present cases the right cerebral hemisphere. It was found convenient to braze a guide on the upper surface of the shield to maintain the intersection of the lead-in conductor and dipole directly over the hole in the shield. In the "C" type of director, which was used throughout, the point of maximum radiation was directly underneath the small screw which held the dipole in place.

The plastic protector of the radiating elements was then placed in contact with the shield and the shield in contact with the scalp over the proposed site. It was found convenient to mark the site lightly with a dot of colored crayon before putting the shield

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[1] Argento, H. F., Centimeter-wave Magnetrons. QST, Dec. 1945.

* Available as the "Microtherm" from the Raytheon Mfg. Co., Waltham, Mass., who courteously supplied the machine with which this work was done.

[2] Osborne, Stafford L., and Frederick, Jesse N., *J.A.M.A.*, 1948.

[3] Krusen, F. H., Herrick, J. F., and Wakim, K. G., *Proc. Staff Meet. Mayo Clin.*, 1947, **22**, 209.

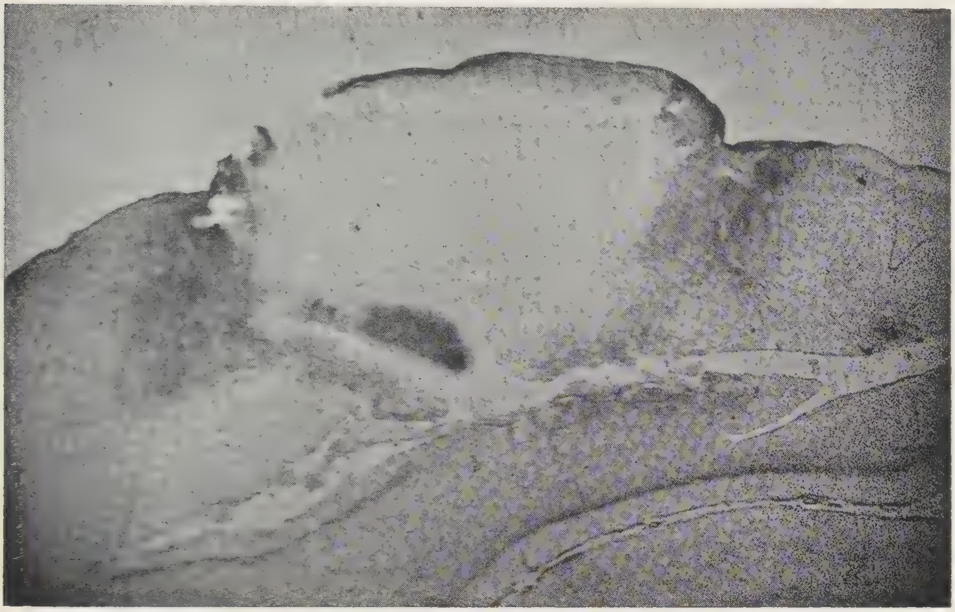

Fig. 1.

Circumscribed area of coagulation necrosis in the cerebral cortex of the rabbit. Nissl's stain. Very low power.

in place. The shield interferes considerably with the efficiency of the radiator but some shielding is necessary lest the ears be seriously burned, since they protrude directly beside the dipole. Prior to irradiation, 30 cc of 5% glucose in water and 100 mg caffeine sodium benzoate were injected intraperitoneally. With the output at 100% the machine was turned on for 3 minutes.

No quantitative temperature readings were taken. They were considered to be grossly inaccurate and misleading since the area of increased temperature within the intracranial cavity was so small.

Results. Seventeen rabbits were used but only 2 representative experiments will be described.

Rabbit 18: The animal received irradiation exactly as described above under "Method." He responded in 30 minutes after irradiation but refused to eat for three days. During this time he received 100 cc 5% glucose in water intraperitoneally daily. Following this he gradually rallied and took food spontaneously. At no time was an asymmetrical motor defect noted. Fifteen days after irradiation the animal was living and well and showed only a crusting lesion of the scalp over the irradiated area. The animal was sacrificed on the 15th day by intracardiac ethyl alcohol. The brain grossly showed a yellow-green coagulation lesion 0.7x1 cm on the superior surface of the posterior portion of the right cerebral hemisphere. The dura was grossly intact and was not adherent to any area of the cortex. Histologically, as seen in Fig. 1, there was a small lesion which appeared sharply circumscribed. It involved the cortical gray matter in its entire width and extended somewhat into the subcortical white matter. The whole lesion contained no intact nerve cells or glia and was delimited by a wall of intensely proliferating glia cells.

Rabbit 16: This animal received irradiation according to the above technic but with elimination of the shield, intraperitoneal fluid and caffeine. The animal did not respond and expired three hours later despite post-irradiation parenteral fluids. Sections of the brain showed a large sloughed area with a slight polymorphonuclear infiltration in the surrounding surviving tissue.

Comment. It appears from the experiments reported that by means of microwave irradiations a focal lesion can be produced in the cerebral cortex of the rabbit without incising the scalp or opening the skull. The only injury to the scalp was a crusting lesion without bleb formation.

A possible explanation of the extremely small size of the lesion is that the plastic protector in contact with a small area of the scalp has dielectric properties which allow much more efficient entrance of the radiation into tissue in the area in contact, than is possible from air to tissue.

The histological lesions produced by this type of irradiation into the cerebral cortex are similar to those described in detail by Silver and Walker[4] in experiments of thermocoagulation of the cerebral cortex by the direct application of a heated piece of metal, except that microwave destruction apparently penetrates more deeply within the tissue.

Since microwave diathermy heating is dielectric heating, it is presumed to be nonionizing and any toxic effects would be those of excessive heating. In fact, no significant hematopoietic effects were observed in the work of Lidman and Cohen,[5] Fallis[6] and Daily[7] upon exposure to intensities of radiation likely to be experienced in practical radar work. It should be noted, however, that Imig, Thomson, and Hines[8] indicate that testicular degeneration may occur from microwave heating at a lower temperature than from infra-red heating, and Richardson, Duane, and Hines[9] showed that upon intentional overdosage, lenticular opacities appeared at about 50°C.

Further work is now in progress using a special butyl rubber impregnated with a titanium compound which allows an intermediate dielectric constant without excessive absorption of the radiation by the rubber. With this technic it is hoped to allow penetration in an area of any shape and, because of the greater efficiency of transfer into the tissue, in a larger area. A modification of this technic may eventually allow the easy destruction of cortical tissue without any surgical procedure both in experimental neurology and in therapeutics.

Summary. A technic is described of producing focal lesions in the cerebral cortex of rabbits by microwave irradiation without incising the scalp or skull. The results on two animals are reported and future possibilities of this work are discussed.

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[4] Silver, M. L., Walker, A. E., *J. Neuropath. Exp. Neur.*, 1947, **10**, 311.

[5] Lidman, B. I., and Cohen, C., *Air Surg. Bull.*, 1945, **2**, 448.

[6] Fallis, R. H., Jr., *Am. J. Physiol.*, 1946, **147**, 281.

[7] Daily, L. E., *U. S. Naval Med. Bull.*, 1943, **41**, 1052.

[8] Imig, C. J., Thomson, J. D., and Hines, H. M., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 383.

[9] Richardson, A. W., Duane, T. D., and Hines, H. M., *J. Neuropath. Exp. Neur.*, 1947, **10**, 311.

Received October 18, 1949. P.S.E.B.M., 1949, **72**.

Ultrafiltration and Ultracentrifugation Studies of Coxsackie Virus. (17459)

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The physical properties of the Coxsackie group of viruses[1–4] have been under investigation since their isolation, and preliminary studies indicated that the original strain is very small. The present report is a summary

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[1] Dalldorf, Gilbert, and Sickles, G. M., *Science*, 1948, **108**, 61.

[2] Dalldorf, Gilbert, Sickles, G. M., Plager, Hildegard, and Gifford, Rebecca, *J. Exp. Med.*, 1949, **89**, 567.

[3] Gifford, Rebecca, and Dalldorf, Gilbert, PROC. SOC. EXP. BIOL. AND MED., 1949, **71**, 589.

[4] Sickles, G. M., and Dalldorf, Gilbert, PROC. SOC. EXP. BIOL. AND MED., 1949, **72**, 30.

of the observations based on filtration through gradocol membranes prepared according to Elford's technic and on sedimentation in the ultracentrifuge. The methods of Bauer and Hughes[5] were used in the filtration experiments, the membranes being sterilized by ultraviolet light. The ultracentrifuge is of the Beams-Pickels type with a rotor of 6-inch diameter. The original strain, T.T., of Coxsackie virus, serologic type 1,[4] was employed in the present work. The legs of infected immature mice and hamsters and the brains of immature mice have served as sources of the virus. The titer of virus in the extremities is markedly higher than that in the central nervous system.[3] For comparison, similar studies were made with brain suspensions of mice infected with MM virus.

Size of Coxsackie Virus. Ten-percent suspensions of the legs of immature hamsters infected with Coxsackie virus and 10% suspensions of MM-infected-mouse brains were prepared in 0.85% salt solution containing 10% infusion broth at pH 7.4. After clarification by centrifugation in a Sorvall angle centrifuge at 8,000 r.p.m. (about 6000 g) for 30 minutes and filtration through a Seitz E-K pad, the suspensions were ultrafiltered on 39 mμ, 33 mμ, and 18 mμ gradocol membranes. The Coxsackie virus suspension, initially having an infectivity titer of approximately 10^{-6}, passed all 3 membranes. Similar results were secured with other Coxsackie virus suspensions prepared from mouse skeletal tissues and brains. Application of Elford's correction factor would indicate that the particle size is 10 mμ or less.

MM virus suspensions similarly tested and treated passed the 39 mμ and 33 mμ membranes and were completely retained by the 22 mμ and 18 mμ membranes. The size of the MM virus estimated from these results would lie between 11 and 16 mμ, which agrees with results reported by other workers[6,7] and indicates that the membranes were satisfactory.

Purification. Suspensions of both viruses have been subjected to ultracentrifugation. It has been reported by Melnick, Shaw, and Curnen[8] that the Coxsackie virus could be largely precipitated after spinning at 31,200 r.p.m. (about 110,000 g) for one hour. In our experience with Coxsackie and MM viruses, much, but not all, of the virus appears in the clear brown pellets. Both viruses have also been purified by the use of methanol according to the method of Pollard.[9] We have used 60% methanol as a precipitating agent and a final concentration of 25%. A 0.2 *M* phosphate buffer at pH 8.0 was used for elution. Satisfactory results were obtained with either virus present as a suspension of infected mouse brain. The recovery of Coxsackie virus from infected immature mouse legs was less satisfactory, perhaps because of the fat present in these suspensions. It is noteworthy that Elford's[10] results indicate that the virus of Newcastle disease is not readily recovered with 20% ethanol at 0°C.

Summary. The original strain of Coxsackie virus, serologic type 1, has been measured using Elford's ultrafiltration technic. Ultracentrifugation furnished a rough check. The virus is very small, the estimated size being 10 mμ or slightly less.

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[5] Bauer, J. H., and Hughes, T. P., *J. Gen. Physiol.*, 1934, **18**, 143.

[6] Jungeblut, C. W., and Dalldorf, Gilbert, *Am. J. Pub. Health*, 1943, **33**, 169.

[7] Gollan, Frank, PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 364.

[8] Melnick, J. L., Shaw, E. W., and Curnen, E. W., PROC. SOC. EXP. BIOL. AND MED., 1949, **71**, 344.

[9] Pollard, Morris, Connolly, Joan, and Fromm, Stanley, PROC. SOC. EXP. BIOL. AND MED., 1949, **71**, 290.

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Received October 17, 1949. P.S.E.B.M., 1949, **72**.

Effects of Administration of Testosterone on Vitamin A-Deficient Rats.

(17460)

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It has been shown by Mason[1] in particular that the effect of vitamin A deficiency on the male rat amounts to a virtual castration. This phenomenon can lead to two main hypotheses. One, vitamin A deficiency may interfere either with the synthesis of testosterone, directly (chemically) or indirectly (*e.g.* secondary effect due to pituitary atrophy in deficient animals). The second probability is that the response of target organs to circulating male sex hormones is affected. To elucidate this point, it was decided to examine and compare the effect of testosterone administration on castrated and non-castrated vitamin A deficient male rats and their controls.

Method. The animals used were male Wistar albino weanlings, 23 days of age. Their average weight was 55 g. Castrated animals had been operated at the age of 21 days and subsequent autopsies showed that all operations had been successful. Prior to the weaning of the experimental animals, their mothers had been put on a vitamin A-poor, "depletion" diet. The animals were kept at room temperature (about 70° F) and were alternately 12 hours in darkness and 12 hours in artificial light. They were fed water and food *ad libitum.* The diet used was the U.S.P. XII diet for vitamin A assay (casein 18%, salt mixture 4%, yeast 8%, starch 65% and vegetable oil 5%) to which was added 100 mg per kg of α-tocopherol. With a yeast-containing diet and a slow growing strain of animals, secondary vitamin C deficiency does not develop as a complicating factor.[2,3] Control animals received orally 1200 I.U. vitamin A every 8 days.*

The animals receiving testosterone were injected every day subcutaneously with 2 mg of testosterone propionate ("Perandren") dissolved in 0.04 cc of sesame oil.† Other animals received a control daily injection of 0.04 cc of pure sesame oil. Thirty-one animals were used. They were divided into the following 8 groups:

a) 6 non-castrates, vit. A deficient
b) 6 non-castrates, vit. A deficient receiving testosterone
c) 3 non-castrates receiving vit. A
d) 3 non-castrates receiving vit. A and testosterone
e) 4 castrates, vit. A deficient
f) 4 castrates, vit. A deficient receiving testosterone
g) 3 castrates, receiving vit. A
h) 2 non-castrates receiving vit. A and testosterone

All animals were weighed every other day. They were sacrificed by decapitation after they had been 64 days on the experiment. By that time most deficient animals showed ocular symptoms of vitamin A deficiency and had lost some weight.

Results and discussion. Table I gives the average weight gains as well as significant average organ weights found. As most groups of animals were too small to permit legitimate statistical treatment and comparison, Table II gives the values of all ratios $\frac{\text{organ weight}}{\text{body weight}}$ found so as to show the distribution of results.

Selye[4] conducted a systematic study of the

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[1] Mason, K. E., *Am. J. Anat.*, 1933, **52**, 153.

[2] Mayer, J., and Krehl, W., *Arch. Biochem.*, 1949, in press.

[3] Mayer, J., *Rev. Can. Biol.*, 1949, in press.

* The authors are indebted to Dr. E. L. Sevringhaus, medical director, Hoffmann-LaRoche, Nutley, N. J., for gifts of vitamins.

† The authors are indebted to Dr. Roy Hertz and to Dr. K. W. Thompson of the Roche-Organon Co., for gifts of "Perandren."

[4] Selye, H., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 142.

TABLE I.
Effect on Body and Organ Weight. Gain in weight during experimental period.

	a. No vit. A No testost.	b. No vit. A + testost.	c. + vit. A No testost.	d. + vit. A + testost.
		1. Non-castrates		
Final* wt gain	44 g	44 g	104 g	84 g
Max. wt gain	54 g	50 g	id.	id.
Testes*	1068 mg	932 mg	2247 mg	1833 mg
Seminal vesicles†	107 mg	1727 mg	193 mg	1749 mg
Kidneys	1149 mg	1448 mg	1769 mg	2187 mg
	e. No vit. A No testost.	f. No vit. A + testost.	g. + vit. A No testost.	h. + vit. A + testost.
		2. Castrates		
Final wt gain	46 g	47 g	104 g	105 g
Max. wt gain	52 g	57 g	id.	id.
Seminal vesicles†	— ‡	1417 mg	— ‡	1720 mg
Kidneys	1083 mg	1560 mg	1517 mg	2429 mg

* The difference between "Final" and "Maximum" gain weights is due to the fact that when the animals were sacrificed, the degree of deficiency reached was such that most animals had already lost weight.

† Prostates and epididymes showed size changes in the same directions as seminal vesicles.

‡ Not detectable by macroscopic examination.

effects of various doses of testosterone on the weight of the body and of certain organs of the male albino rat. He found particularly striking effects for doses of 1.0 to 10 mg daily. This is an analogous dosage to that used here. The experiment was continued for only 20 days—approximately one-third of the duration of the present experiment. In these conditions, he observed that accessory sex organs, the seminal vesicles in particular were very much enlarged, while the size of the testes was decreased. In this particular series of his no significant effect was observed on the weight of the kidneys, in contradistinction with some other of his own observations[5-7] and those of other investigators.[8-10]

In the present work, the deficient animals, castrates as well as non castrates conformed to the general pattern established and verified for the control animals: testosterone administration was followed by extreme hypertrophy of accessory sex organs, seminal vesicles in particular; by a definite hypertrophy of the kidneys and by a decrease of the size of the testes. As far as the effect on the overall body weight is concerned, while the decrease in weight gain of normal animals under the influence of testosterone administration noted by Selye was confirmed, deficient animals behaved like castrates in that they were not affected.

Particularly significant is the fact that the extreme hypertrophy of accessory sex organs was maintained by testosterone even in the face of acute deficiency. Animals showing marked periocular symptoms and which had sustained severe losses of weight extending over a period of two weeks still showed seminal vesicles, prostates and epididymes 10 times larger, in absolute values, than control animals not receiving testosterone. This differential reaction is in itself quite striking.

This experiment seems to establish definitely the fact that any "castration effect" observed as a result of vitamin A deficiency in the rat is not due to the lack of response of target organs but to lack of circulating androgens. Because of previous results indicating the apparent "piling up" of cholesterol, in Vitamin A deficiency,[11-13] a possible explana-

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[5] Selye, H., *J. Urol.*, 1939, **42**, 637.

[6] Selye, H., *Canad. Med. Assn. J.*, 1940, **42**, 113.

[7] Selye, H., *J. Endocrinol.*, 1939, **1**, 208.

[8] Korenchevsky, V., and Ross, M. A., *Brit. Med. J.*, 1940, **1**, 645.

[9] Paschkis, K. E., Shay, H., Gershon-Cohn, J., and Fels, S. S., *Am. J. Physiol.*, 1940, **129**, 191.

[10] Pfeiffer, C. A., Emmel, V. M., and Gardner, W. U., *Yale J. Biol. Med.*, 1940, **12**, 493.

[11] Ralli, E. P., and Waterhouse, A., PROC. SOC. EXP. BIOL. AND MED., 1933, **30**, 519.

TABLE II.

$\frac{\text{Organ weight}}{\text{Body weight}}$ Relationship. Distribution of Values.

	a. No vit. A No testost.		b. No vit. A + testost.		c. + vit. A No testost.		d. + vit. A + testost.	
			Non-castrates					
$\frac{\text{Testes}}{\text{body}} \times 10^3$	10.4	11.5	8.5	10.8	14.3	14.2	12.9	13.4
	11.2	9.8	8.5	12.2	14.4		14	
	10.2	12.8	10.8	8.5				
	Avg	11	Avg	9.9	Avg	14.3	Avg	13.4
$\frac{\text{Seminal vesicles}}{\text{body}} \times 10^3$	0.002	1.78	15	18.4	1.7	0.8	10	12.5
	0.64	0.8	17.2	23.2	1.4		17.8	
	2	1.47	17.7	18.8				
	Avg	1.1	Avg	18.4	Avg	1.2	Avg	13.4
$\frac{\text{Kidneys}}{\text{body}} \times 10^3$	14.7	15	13.6	15	13.4	9.8	16.3	16
	13.8	12.9	19.8	14.8	10.5		14.3	
	13.3	12.2	13.4	13.2				
	Avg	13.7	Avg	15.0	Avg	11.2	Avg	15.6
	e. No vit. A No testost.		f. No vit. A + testost.		g. + vit. A No testost.		h. + vit. A + testost.	
			Castrates					
$\frac{\text{Seminal vesicles}}{\text{body}} \times 10^3$	—		12.9	12.5	—		10.3	10.9
			13.1	12.7				
			Avg	12.8			Avg	10.6
$\frac{\text{Kidneys}}{\text{body}} \times 10^3$	12.1	12.8	14	14.2	9.3	9.2	14.2	15.8
	14.1	13.4	14.1	14.4	9.5			
	Avg	13.1	Avg	14.2	Avg	9.3	Avg	15.0

tion is that vitamin A deficiency prevents chemical transformation of cholesterol into testosterone. Indirect mechanisms, such as intervention of vitamin A in the synthesis or release of gonadotrophic hormones cannot, however, be eliminated without further experimental work.

Summary. 1. In an effort to find whether the "virtual castration" of vit. A deficient male rats is due to prevention of the synthesis of androgens or to the lack of response of target organs, the effects of administration of testosterone in deficient non-castrates and castrates were compared with the effects on their controls.

2. The response of the deficient animals was found to be essentially normal. The accessory sex organs in particular were extraordinarily hypertrophied under the influence of testosterone administration even in the face of severe losses of weight.

3. The evidence obtained suggests that vitamin A deficiency interferes with the synthesis or release of androgens.

Received August 4, 1949. P.S.E.B.M., 1949, **72**.

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[12] Smith, M. E., *J. Nutrition*, 1934, **8**, 675.

[13] Mayer, J., unpublished observations.

Blood Histamine, Leukocytes and Platelets in Experimental Serum Disease in Rabbits. (17461)

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The role of histamine in allergic diseases is poorly understood. During anaphylactic shock the blood histamine level has been found to be markedly elevated in dogs and guinea pigs immediately following the reinjection of antigen.[1-5] It is believed that this elevation can account for practically all dominant symptoms of this condition except the incoagulability of the blood which is explained by outpouring of heparin.[5] However, in rabbits, horses, and calves the blood histamine level has been shown to be depressed.[5,6] In rabbits this deviation can plausibly be explained by liberation of histamine from the buffy coat which in these animals contains 200 γ of histamine per 100 cc of blood as compared with 2 to 4 γ in dogs and 12 to 25 γ in guinea pigs.[5] The depression of the blood histamine in horses and calves is difficult to explain on this basis for these animals contain only 2 to 4 γ of histamine per 100 cc of blood.

The role of histamine in serum disease has been studied by several investigators. Dammin and Bukantz[7] were unable to produce the vascular lesions of this disease in normal rabbits by giving them daily intravenous injections of 0.1 mg of histamine base per kg of body weight for periods varying from 1 to 62 days. Injecting histamine into the rabbit's skin seldom caused erythema or whealing, and the site of injection, unlike that of an Arthus phenomenon, generally did not fluoresce following the intravenous injection of fluorescein. Moreover, in rabbits injected with horse serum, neohetramine given subcutaneously in doses of 10 mg per kg twice daily did not affect the appearance of circulating antibodies, the Arthus reaction of the skin or the development of arteritis.[7] Benadryl in doses of 5 mg per kg every 8 hrs was likewise ineffective.[8]

However, the latter drug in doses of 2 or 10 mg twice daily, and drug 1627, another antihistaminic agent, have been reported to impede the development of the myocardial lesions of serum disease,[9] and benadryl in doses of 5 mg per kg every 8 hrs apparently offered some protection against the production of lesions in the valves.[8] These results have not been duplicated.[7,8] Antistine has been found to prevent experimental glomerulonephritis in rabbits produced by anti-kidney serum,[10] but the glomerulitis of experimental serum disease was not affected by antihistaminics.[11]

In view of these various findings it seemed of interest to study the blood histamine level in experimental serum disease, and as the histamine of the blood is known to be contained in the buffy coat, to investigate the leukocytes and platelets as well.

Material and Methods. All experiments were performed in Chinchilla rabbits averaging 1900 g in weight. Twenty-five rabbits were given 15 cc per kg of "normal horse serum" (Squibb) containing 1:10000 sodium ethyl mercuri thiosalicylate plus 0.2% phenol. The remaining 15 rabbits received horse

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[1] Dragstedt, C. A., and Mead, F. B., *J. Immun.*, 1936, **30**, 319.

[2] Dragstedt, C. A., and Mead, F. B., *J. Pharm. and Exp. Therap.*, 1936, **57**, 419.

[3] Simon, A., and Staub, A. M., *Compt. rend. Soc. de biol.*, 1937, **125**, 815.

[4] Code, C. F., *Am. J. Physiol.*, 1939, **127**, 78.

[5] Dragstedt, C. A., *Physiol. Rev.*, 1941, **21**, 563.

[6] Code, C. F., and Hester, H. R., *Am. J. Physiol.*, 1939, **127**, 71.

[7] Dammin, G. J., and Bukantz, S. C., *J. Am. Med. Assn.*, 1949, **139**, 358.

[8] Roberts, R. C., Crockett, K. A., and Laipply, T. C., *Arch. Int. Med.*, 1949, **83**, 48.

[9] Kyser, F. A., McCarter, J. C., and Stengle, J., *J. Lab. and Clin. Med.*, 1947, **32**, 379.

[10] Reubi, F., *Helvet. Med. Acta*, Suppl. 18, 1946.

[11] Janeway, C. A., oral communication, 1949.

serum without preservative (Wyeth).* Seventeen days later the surviving animals were desensitized with 1 cc of serum intravenously and 2 days thereafter they were reinjected with another 15 cc per kg. Twenty-four rabbits received serum only, 16 were given 20 mg of neohetramine in a 4% solution subcutaneously 4 times daily (at 9 AM and at 1, 5 and 9 PM). The injections of the drug were discontinued 3 days after reinjection of the serum, *i.e.*, 4 days before the last 4 rabbits receiving the drug were sacrificed. Histamine was determined according to Code's method.[12] The blood was collected from the heart in quantities of 10 cc. In order to avoid a possible change in the blood histamine level by repeated bleeding, blood was drawn as a rule only once or twice from the same animal except in a last series of 3 rabbits which were bled 5 times each. The dates of collection are indicated in Fig. 1. Leukocyte and platelet counts were first done on certain days only, but in our last series of 3 rabbits 12 determinations were made in each animal. All counts were made shortly before blood was collected for histamine determination. They were made on blood obtained by nicking the marginal vein of the ear with a sharp razor blade. Groups of 4 animals were sacrificed and studied microscopically 3, 4, 5, 7, 14 and 16 days after the first injection of serum, and 2, 6 and 7 days after the second.

Results. The Arthus reaction of the skin elicited by intradermal injection of 0.1 cc of horse serum was, as in the experiments of Dammin and Bukantz,[7] not appreciably changed by neohetramine in 20 rabbits tested. Analysis of the urine at the time of sacrifice showed no significant difference between the rabbits receiving neohetramine and the controls during the later stages of the experiment. However, the proteinuria which occurred during the week following the first injection of horse serum was abolished by the antihistaminic (Table I). The histamine level of the blood before injection averaged 194 γ per 100 cc in 6 rabbits tested. After the intravenous injection of 15 cc of horse serum per kg the level rose to an average of 226 γ on the 4th day and then gradually returned to normal (Fig. 1). Reinjection of serum on the 19th day was followed by a sharp drop in histamine to an average of 182 γ on the 1st day. There was no elevation of this material during the first 7 days after the second injection.

The rabbits which received neohetramine revealed an average blood histamine level of

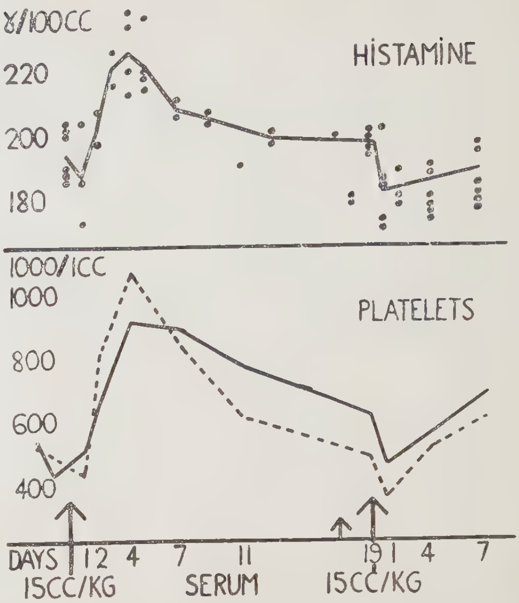

FIG. 1.
Histamine and platelet levels in serum disease. The large arrows indicate the first and second injection of 15 cc of horse serum per kg, the small arrow the injection of the desensitizing dose of 1 cc.

TABLE I.
Protein Content of Urine Following the First Injection of Serum.

Days after inj.	Without	With
	Neohetramine	
4	+	0
	+ −+	0
5	+	0
	+	0
7	(+)	(+)
	(+)	0
14	(+)	0
	0	0

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* We are indebted to Dr. J. Seifter of the Wyeth Institute of Applied Biochemistry for supplying us with the neohetramine solution, and Dr. B. Scott Fritz and Dr. J. H. Brown of the Wyeth Incorporated Biological Laboratory at Marietta, Pa., for giving us the horse serum.

[12] Code, C. F., *J. Physiol.*, 1937, **89**, 257.

TABLE II.

Leukocyte and Platelet Levels Following the First and Second Injection of Serum in Rabbits No. 1, 2, 3.

Days after inj. Rabbit No.	Neutrophiles			Lymphoid cells			Eosinophiles			Basophiles			Platelets (thousands)		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Before inj.															
	1622	2967	2342	4400	3349	4314	31	0	0	187	64	136	543	524	756
	1228	1872	2630	4182	4017	4558	30	30	37	140	121	185	427	499	685
After first inj.															
1	1408	988	2442	4847	2660	4514	131	95	74	164	57	370	511	428	533
2	4493	1084	1588	7128	3729	4027	60	25	0	300	202	265	660	817	475
4	3640	6837	3220	3100	4596	3187	0	0	0	0	57	133	908	1068	701
7	2784	3559	4232	5193	5703	3976	42	49	0	291	439	342	898	850	936
11	3353	2550	5408	3724	3858	7452	112	0	0	261	302	330	774	610	670
19	5490*	1404	1350	5270*	3591	7850	0	27	0	330	378	800	613	483	1064
After second inj.															
1	4085*	1564	7709	2074	1717	2399	31	17	0	0	102	102	458	357	727
4	1952	1748	2138	8991	7343	3680	59	50	0	828	849	1192	562	524	669
7	1639	2275	2984	6423	4302	3581	89	71	37	709	462	858	682	609	790

* This elevation is accounted for by a retro-aural abscess which developed in this rabbit.

73 γ per 100 cc (42 to 102) from the 3rd to the 17th day after the first injection, and 72 γ after the second. The blood for these determinations was drawn mostly 4 hrs after the last injection of neohetramine.

The results of the leukocyte and platelet counts in the 3 most completely studied rabbits are given in Table II and Fig. 1. It is apparent that the first injection of serum caused first a rise in eosinophiles (on the 1st day) which was followed by a rise in neutrophiles (between the 2nd and 4th day) and lymphoid cells (on the 4th day) whereas the basophiles were not much changed. While 2 of the rabbits behaved much alike with the exception of a high neutrophile count on the 19th and 20th day in Rabbit 1 (caused by a retro-aural abscess), and while most of our counts in other rabbits fell well within the range of the counts of these 2 animals, Rabbit 3 behaved differently showing a peak in neutrophiles on the 11th day and one in lymphoid cells between the 11th and 19th day. At autopsy this animal revealed the most severe arteritis of all animals of this series; it also showed a marked gomerulitis though this was less severe than that in Rabbit 2.

The reinjection of serum on the 19th day caused a marked drop in lymphoid cells and basophiles one day after injection. This was followed by a marked rise of the 2 with a peak on the 4th day. The average rise in lymphocytes was 178% (161% over the normal control counts), that in basophiles 1300% (590% over the normal control counts). The neutrophiles showed no consistent change.

The platelets were markedly elevated following the first injection of serum with a peak on the 4th day; they showed a marked drop one day after the second injection and thereafter returned to normal (Fig. 1). Rabbit 3 differed again from this course, whereas most of our counts in other rabbits fell well within the range of counts of Rabbits 1 and 2. The course of the platelets closely paralleled that of histamine.

The important morphological changes in our rabbits are given in Table III. It can be seen that the rabbits which were treated with serum containing preservative showed more pulmonary involvement and less myocarditis, glomerulitis and arteritis than those receiving serum without preservative. This difference may have been due to precipitation and therefore increased retention in the pulmonary vessels of the preservative containing serum, for sections of these lungs showed numerous emboli blocking arterioles and capillaries of the lungs, whereas those of animals receiving serum without preservative did not. The chief difference in the reactions toward the

TABLE III.

Frequency (a)* and Intensity (b)† of the Various Lesions Caused by Serum.

No. of inj. of serum	Neohetramine	No. of rabbits	Mesenchymal myocardium		Reactions, lungs		Glomerulitis		Valvulitis		Arteritis	
			a.	b.	a.	b.	a.	b.	a.	b.	a.	b.
Serum with preservative												
1	with	8	62.5	52.5	100	48.5	0	0	0	0	0	0
	without	8	50	52.5	100	83	12.5	17	0	0	0	0
2	with	3	100	57.5	100	100	33.3	33	66.7	25	0	0
	without	3	100	62.5	100	92	33.3	17	50	100	33.3	50
	” ‡	6	100	75	100	71	66.7	21	40	83	16.7	50
Serum without preservative												
1	with	3	100	21	66.7	31	0	0	0	0	0	0
	without	4	100	44	100	34.5	25	17	0	0	0	0
2	with	2	100	75	100	50	100	54	100	83	50	50
	without	2	100	31	100	56	50	50	—	—	50	75
	” ‡	6	100	39.5	100	71	83.3	67	40	67	50	67

* The frequency has been expressed in per cent of animals affected in order to facilitate comparison.

† The intensity has been expressed in per cent of the maximum intensity observed. The figures were calculated from the positive cases only.

‡ These animals were members of separate groups not receiving neohetramine.

first and second injection of serum was a greater frequency and intensity particularly of glomerulitis, valvulitis and arteritis following the latter. In fact, valvulitis and arteritis occurred in these experiments only after the second injection. The mesenchymal reactions in lungs and myocardium were more severe, too, though less strikingly so, as may be expected if our contention is correct that these reactions are chiefly primary reactions instrumental in the production of antibody, whereas glomerulitis and arteritis are chiefly allergic in nature, *i.e.*, the result of an antigen-antibody reaction.[14,15]

Neohetramine was found to depress the mesenchymal reactions in lungs and myocardium. This effect was most noticeable in the organs whose mesenchyme reacted most severely to the serum, *i.e.*, in the lungs, in the rabbits treated with serum containing preservative, and in the lungs and myocardium, in the animals which were given serum without preservative. Also the 2 cases of glomerulitis observed after the first injection of serum occurred both in animals without neohetramine. Following the 2nd injection no significant difference was observed between the two series of experiments. However, the antihistaminic drug was discontinued 4 days before the last animals were sacrificed.

Discussion. The first intravenous injection of a large dose of horse serum in our rabbits caused a rise in histamine and platelets, the peak occurring on the 4th day. The reinjection of the same quantity of the same serum 19 days later was followed by a marked drop of the two elements during the first day, and there was no rise above normal during the remaining 6 days of the experiments. The rise in platelets after the first injection resembled that following various infections, the exposure to ultraviolet light, and other injuries,[16] while the drop in histamine after reinjection was in accord with the observed fall of histamine in anaphylactic shock in rabbits.[5] The rise in platelets following the first injection and the absence of a rise after reinjection furnish an explanation for the recent observation of increased blood coagulability during the first week following the first injection of serum and its absence after the second.[17] The close parallel between histamine and platelets in our rabbits (Fig. 1) is in keeping with the demonstration by Minard[18] and others that in rabbits most of the blood histamine is contained

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[14] Ehrich, W. E., Seifter, J., and Forman, C., *J. Exp. Med.*, 1949, **89**, 23.

[15] Forman, C., Seifter, J., and Ehrich, W. E., *J. Allergy*, 1949, **20**, 273.

[16] Gunn, F. D., *Arch. Path.*, 1931, **12**, 153.

[17] Moore, R. C., and Waugh, D., *J. Exp. Med.*, 1949, **89**, 541.

in the platelets.

It is obvious that the rise in histamine and platelets following the first injection of serum cannot be regarded as an allergic phenomenon; it is apparently part of the primary reaction of the organism against noxious agents in general. During the period of histamine and platelet elevation following the first injection of serum all rabbits tested which received serum only contained protein in the urine, whereas, those which were given neohetramine did not. Though one may be tempted to explain this as a specific antihistaminic effect, there is reason to believe that this was due to a non-specific action of the drug upon the permeability of the capillaries possibly via the hyaluronidase-hyaluronic acid mechanism; for it has been found that antihistaminic drugs decrease the permeability of tissue membranes *in vitro*[19] as well as that of capillaries *in vivo*,[20,21] and it has also been observed that hyaluronidase enhances the leakage of protein through the glomeruli in serum disease in rabbits.[22] However this may be, microscopic examination of the kidneys showed that the proteinuria in our animals was not caused by proliferative glomerulitis, but it was associated with degenerative changes such as swelling of the loops and pyknosis of the nuclei as described and illustrated in a previous paper.[14]

The mesenchymal reactions in the lungs following the first injection of serum were distinctly, and those of the myocardium slightly depressed by neohetramine. The latter observation is in accord with the findings of Kyser and coworkers.[9] The depressing effect of neohetramine, if real, is opposite to that of hyaluronidase[22] and therefore may well be explained by a non-specific action of this drug on the permeability of tissue membranes causing diminished penetration of lungs and myocardium by antigen. The development of arteritis, glomerulitis and valvulitis following reinjection, on the other hand, was not affected. These observations are in keeping with our view that the mesenchymal reactions in lungs and myocardium are direct reactions to the antigen instrumental in the production of antibody, whereas arteritis and glomerulitis are the result of an antigen-antibody reaction.[14,15]

Of the changes of the leucocytes, the marked rise in basophiles following the second injection of serum is of particular interest. As the reinjection is followed by outpouring of heparin,[5] and there is reason to believe that the basophiles are cellular sources of heparin,[23] it suggests itself that the rise in basophiles may be a regenerative phenomenon prompted by the heparin consuming effect of the anaphylactic reaction.

Conclusions. It appears that in rabbits histamine is linked via the histamine containing platelets with blood coagulation, and this is connected via heparin with the heparin producing basophiles. All these elements are involved in serum disease. The first injection of serum is followed by a rise in histamine and platelets, the second by a drop in these elements. The destruction of the white cell layer following reinjection calls forth an outpouring of heparin which in turn is followed by basophilia in the blood. Only the changes following reinjection can be regarded as allergic. Neohetramine was found to effect only the primary reactions following the first administration of serum. It suppressed the rise in blood histamine and the proteinuria observed during the first week following the first injection, and also impeded the primary mesenchymal reactions in lungs and heart which are believed to be instrumental in antibody formation. There are reasons to believe that this was not a specific antihistaminic effect, but that it was due to a non-specific action of the drug upon the permeability of tissue membranes possibly via the hyaluronidase-hyaluronic acid mechanism.

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[18] Minard, D., *Am. J. Physiol.*, 1941, **132**, 327.

[19] Seifter, J., Baeder, D. H., and Dervinis, A., PROC. SOC. EXP. BIOL. AND MED., 1949, **72**, 136.

[20] McGavack, T. H., Elias, H., and Boyd, L. J., *Am. J. Med. Sc.*, 1947, **213**, 418.

[21] Leger, J., and Masson, G., *ibid.*, 1947, **214**, 305.

[22] Seifter, J., and Ehrich, W. E., to be published.

[23] Jorpes, J. E., Heparin, Oxford University Press, 1946.

Received Sept. 27, 1949. P.S.E.B.M., 1949, **72**.

Detoxification of Cottonseed Pigment Glands with Ferrous Sulfate. (17462)

EDWARD EAGLE (Introduced by A. J. Carlson)

From the Research Laboratories, Swift and Co., Chicago, Ill.

The use of iron salts as antidotes for cottonseed meal injury was suggested many years ago by Withers *et al.*[1] who successfully fed ferric ammonium citrate-treated cottonseed meal to rabbits and ferrous sulfate and ferric chloride-treated meal to pigs. In a recent paper[2] we studied the acute oral toxicity on rats, mice, rabbits and guinea pigs of cottonseed pigment glands, distinct morphological structures recently made available from cottonseed kernels by a flotation process.[3] The present report concerns LD50 studies on rats of different samples of pigment glands each of which was administered in both distilled water and in 2% ferrous sulfate solution.

Procedure. Male rats weighing between 150 and 220 g were fasted for 18 hours (average weight loss from fasting was 14 g). Water was given *ad libitum*. Each rat was kept in a separate cage in an air-conditioned animal room maintained at 80° ± 1°F and ca. 45% relative humidity. For each of the 3 samples the test preparations were made both by mixing 10 g of the pigment glands with distilled water to a final volume of 100 ml, and by suspending 10 g of the same glands in 2% ferrous sulfate solution to a final volume of 100 ml. Single doses were given by stomach tube on the basis of milligrams of pigment glands per kilogram body weight. All animals had free access to stock diet and water after dosing. Calculations of the LD50

TABLE I.
Mortality in Rats after Oral Administration of Single Doses of Cottonseed Pigment Glands in Water and in Ferrous Sulfate Solution.

		Sample 1		Sample 2		Sample 3		
	Dose, mg/kg	In water	In 2% $FeSO_4$	In water	In 2% $FeSO_4$	In water	In 2% $FeSO_4$	In 0.5% $FeSO_4$
	7000		0/4		0/4		0/1	4/4
	6000		0/4		0/4		0/4	4/4
	5400				0/4		0/4	
	5000		0/4		0/4		0/4	4/4
	4600					4/4	0/4	
	4200					5/7	0/4	
	4000		0/4		0/4	3/4		4/4
	3800					6/7	0/4	
	3400		1/4		0/4	4/4	0/5	4/4
	3000		0/4		0/4	8/11	0/5	4/4
	2600	3/3	0/4	4/4		3/3		
	2400			4/4				
	2200	3/4	0/4	4/4		6/11		
	2000			4/4				
	1800	7/7	0/4	3/4		4/4		
	1600			3/4				
	1400	7/8		2/4		0/8		
	1200	2/4		0/4				
	1000	3/7						
Total rats used		33	36	32	28	63	36	24
LD50 (mg/kg)		1140	>7000	1490	>7000	2290	>7000	<3000

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[1] Withers, W. A., and Brewster, J. F., *J. Biol. Chem.*, 1913, **15**, 161; Withers, W. A., and Carruth, F. E., *ibid.*, 1917, **32**, 245.

[2] Eagle, E., Castillon, L. E., Hall, C. M., and Boatner, C. H., *Arch. Biochem.*, 1948, **18**, 271.

[3] Boatner, C. H., and Hall, C. M., *Oil and Soap*, 1946, **23**, 123; Vix, H. L. E., Spadaro, J. J., Westbrook, R. D., Croveto, A. J., Pollard, E. F., and Gastrock, E. A., *J. Am. Oil Chemists' Soc.*, 1947, **24**, 228.

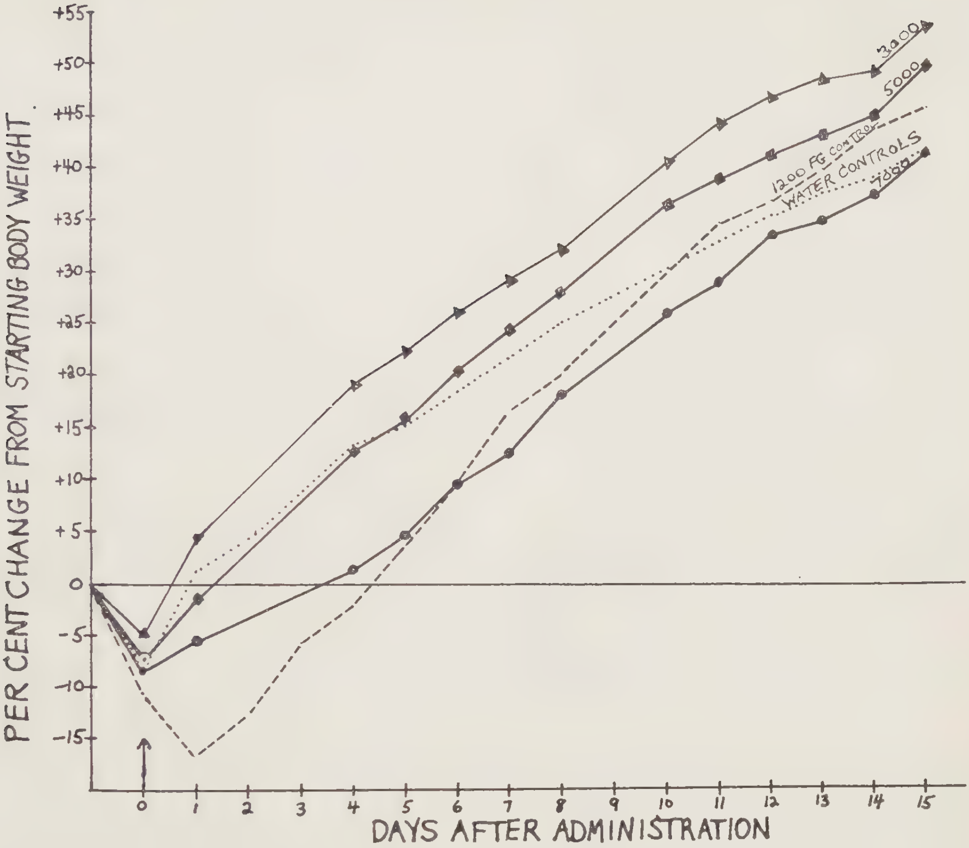

FIG. 1.

Effect on the body weight of the rat of large, single doses of cottonseed pigment glands detoxified by administration in 2% ferrous sulfate solution.

values were made by the method of Reed and Muench.[4]

Results. A summary of the experimental data is given in Table I. Doses of all 3 samples of cottonseed pigment glands which caused 100% mortality when administered in distilled water had no harmful effect when given in 2% ferrous sulfate solution. In the case of Sample 3, cottonseed pigment glands which were completely detoxified when given in 2% $FeSO_4$ lost none of their toxicity when suspended in 0.5% $FeSO_4$ and all of the rats died. The rapid onset of diarrhea reported for cottonseed pigment glands[2] occurred at every dose level when distilled water was the vehicle, but no diarrhea occurred when the glands were suspended in 2% ferrous sulfate solution. All fatalities except one occurred within 3 days, 82% within 24 hours. Typical body weight curves obtained on rats after administration of unusually large single doses (indicated by arrow) of cottonseed pigment glands (Sample 2) are illustrated in Fig. 1. Each point (solid lines) represents an average value obtained from 4 rats. The water control rats (dotted line) received water only; the pigment gland control rats (dashes) received 1200 mg/kg of the glands suspended in water. Since the LD50 value for these pigment glands was 1490 mg/kg, all of the experimental rats would have died had these large doses (3000 to 7000 mg/kg) been administered in distilled water instead of in 2% $FeSO_4$ solution. The 1200 mg/kg dose of pigment glands in water caused a marked depression in body weight; the large doses administered in 2% $FeSO_4$ caused no decreased weight beyond the fasting level. Similar body weight curves were obtained with 22 other dose levels of the 3 samples of cottonseed pigment glands.

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[4] Reed, L. J., and Muench, H., *Am. J. Hygiene*, 1938, **27**, 493.

Summary. Acute toxicity studies were made on 3 different samples of cottonseed pigment glands administered orally to 228 rats in the form of slurries made with either distilled water or 2% ferrous sulfate solution. The LD50 values for the samples when administered in water were, respectively, 1140, 1490 and 2290 mg/kg. When given in 2% ferrous sulfate solution the samples were detoxified so that even such high doses as 7000 mg/kg for each were no longer toxic. There was little effect on the body weight of all 99 rats which survived doses of ferrous sulfate-treated cottonseed pigment glands 3 to 6 times the LD50 value for water-suspended glands. Administration of pigment glands in 0.5% $FeSO_4$ did not detoxify them, and led to the death of all 24 rats given this preparation.

The author is indebted to Mr. H. F. Bialek for technical assistance, and to the Southern Regional Laboratory, U.S.D.A., New Orleans, La., for the samples of cottonseed pigment glands.

Received October 18, 1949. P.S.E.B.M., 1949, **72**.

Effect of 3-Ortho-toloxy-1, 2-propanediol (Tolserol, Myanesin) upon Electrogram of Human Cortex and Subcortex. (17463)

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The sites of action of 3-ortho-toloxy-1,2-propanediol (tolserol, myanesin) have not been exactly determined as yet, although there is suggestive evidence that this drug chiefly affects subcortical ganglia (Berger and Bradley;[1] Burke and Linegar;[2] Davison;[3] Pugh and Enderby;[4] Schlesinger *et al.*[5]). Particularly the experience that it is able to abolish petit mal discharges (Gammon and Churchill[6]) points to a thalamic effect, since these discharges seem to originate in thalamic nuclei (animal experiments of Jasper,[7] human records of Wycis, Lee and Spiegel[8]).

A study of the effect upon the electrical discharges of subcortical ganglia promised to supply more direct evidence. In the course of thalamotomy and mesencephalotomy operations carried out by means of our stereoencephalotome (Spiegel and Wycis[9]), we had an opportunity to record the potentials of human diencephalic and mesencephalic structures preceding the production of the lesions.

The effect of tolserol upon the electroencephalogram (scalp electrodes in the frontal and in the occipital region), upon the electrical discharges of thalamic nuclei (dorsomedial nucleus; lateral nucleus), of the hypothalamus, and of the tectum of the midbrain was studied. Needle electrodes consisting of an inner stylet, insulated except for the tip, and an outer sheath insulated except for a ring 3.0 mm above the tip of the stylet were inserted by means of the stereoencephalotome. Monopolar as well as bipolar leads were used. For monopolar recording, the tip of the inner

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[1] Berger, F. M., and Bradley, W., *Brit. J. Pharm.*, 1946, **1**, 265.

[2] Burke, J. C., and Linegar, C. R., *Fed. Proc.*, 1948, **7**, 208.

[3] Davison, W. H. A., *Brit. Med. J.*, 1948, **1**, 544.

[4] Pugh, J. I., and Enderby, G. E. H., *Lancet*, 1947, **2**, 387.

[5] Schlesinger, E. B., Drew, A. L., and Wood, B., *Am. J. Med.*, 1948, **4**, 365.

[6] Gammon, G. D., and Churchill, J. A., *Am. J. Med. Sc.*, 1949, **217**, 143.

[7] Jasper, H. H., and Droogleever-Fortuyn, J., *Res. Publ. Assn. Nerv. Ment. Dis.*, 1947, **26**, 272.

[8] Wycis, H. T., Lee, A. J., and Spiegel, E. A., *Conf. Neur.*, 1949, **9**, 264.

[9] Spiegel, E. A., Wycis, H. T., Freed, H., and Lee, A. J., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 175.

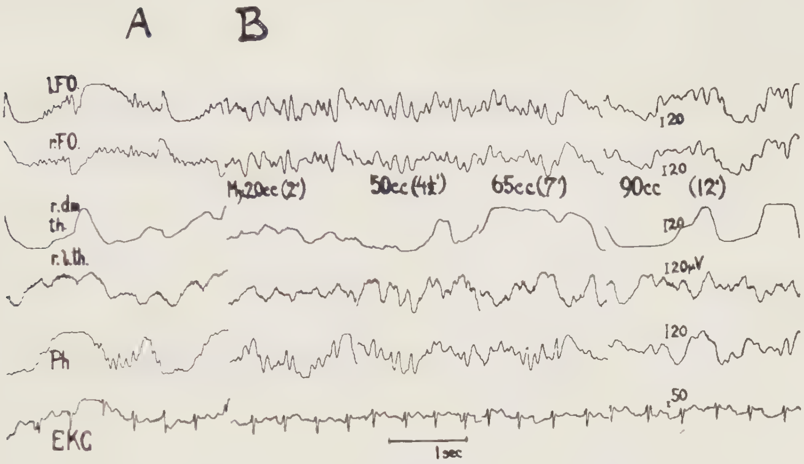

FIG. 1.

Effect of intravenous injection of 2% Tolserol solution in a schizophrenic patient chiefly upon the EEG (1FO bipolar record from left frontal and occipital scalp electrodes, rFo bipolar record from right frontal and occipital scalp electrodes). No definite effect upon the dorsomedial nucleus (r. dm. th) and the lateral nucleus (r l th) of the right thalamus. No definite changes in the pharyngogram (Ph) and the electrocardiogram (EKG). A large part of the right dorsomedial nucleus is degenerated by a previous thalamotomy. A before, B after injection of Tolserol (Myanesin My). Time after injection is indicated in brackets. Anesthesia: .6 mg Atropine sulf.; 30 cc Paraldehyde; Demerol 37.5 mg; 0.5 g Sod. Pentothal; N_2O-O_2 (3:1).

stylet served as the active, the lobe of the homolateral ear as the "indifferent" electrode. For bipolar recording the tip of the stylet served as the one and the bare ring on the sheath as the 2nd electrode. Simultaneously the electrocardiogram and the electrical discharges lead off from the roof of the pharynx were recorded. These records were obtained in schizophrenic patients, cases of depression and obsessive compulsive neuroses preceding thalamotomy and in cases of unbearable pain preceding mesencephalotomy. All patients were under nembutal or pentothal, N_2O-O_2 anaesthesia; 50-100 cc 2% Tolserol* were injected intravenously.

The following changes of the electrical activity were observed: In some instances, the fast, low voltage oscillations of the EEG of the anesthetized patients were replaced by slower waves of higher amplitude (Fig. 1). The speed of the injection seems of importance, if one wishes to demonstrate alterations of the EEG. We observed the above described effect, if the first 50 cc of the drug were injected within 4-5 minutes and the following 50 cc within 6-8 minutes. In contrast to the susceptibility of the cerebral cortex to tolserol, we failed to obtain definite changes of the EEG following the injection of curare in doses sufficient to produce peripheral paralysis. This latter observation is in agreement with the findings of Harvey,[10] Everett,[11] and Girden.[12] While no definite changes in the discharges of the dorsomedial nuclei (dm th) and of the lateral nuclei (lat th) of the thalamus were found (Fig. 1), the hypothalamic lead (Hm and Hb) showed a definite depression of the discharges interrupted by the appearance of burst of slow waves of high amplitude (Fig. 2). These changes appeared at a stage when a cortical effect was not definite as yet. The same applies to the tectum of the midbrain whose fast, low voltage discharges were replaced by high 3-5/second waves (Fig. 3).

Summary: Tolserol has a definite effect

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* Kindly supplied by Dr. H. Sidney Newcomer, E. R. Squibb and Sons.

[10] Harvey, A. M., *Fed. Proc.*, 1948, **7**, 458.

[11] Everett, G. M., *J. Pharm. and Exp. Therap.*, 1948, **92**, 246.

[12] Girden, E., *J. Neurophysiol.*, 1948, **11**, 169.

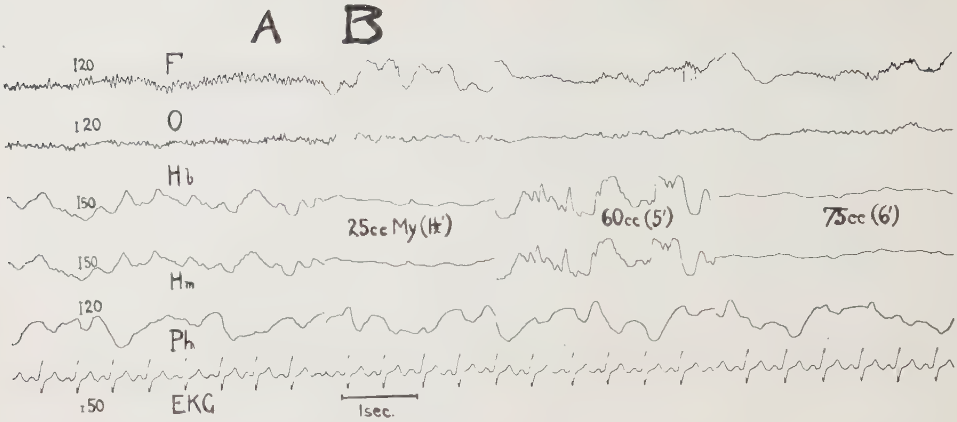

FIG. 2.

Effect of intravenous injection of 2% Tolserol solution (My) chiefly upon the hypothalamogram in a paranoid schizophrenic. Hb bipolar, Hm monopolar records from the hypothalamus. Note that the pharyngogram does not reflect the changes in the hypothalamogram. F bipolar record from the frontal areas, O from the occipital areas (scalp electrodes) A before, B after injection of the drug. Anesthesia: Atropine sulf. 0.4 mg; Sod. Phenobarbital 0.2 g; Sod. Pentothal 1.3 g; Curare 100 units; N_2O-O_2.

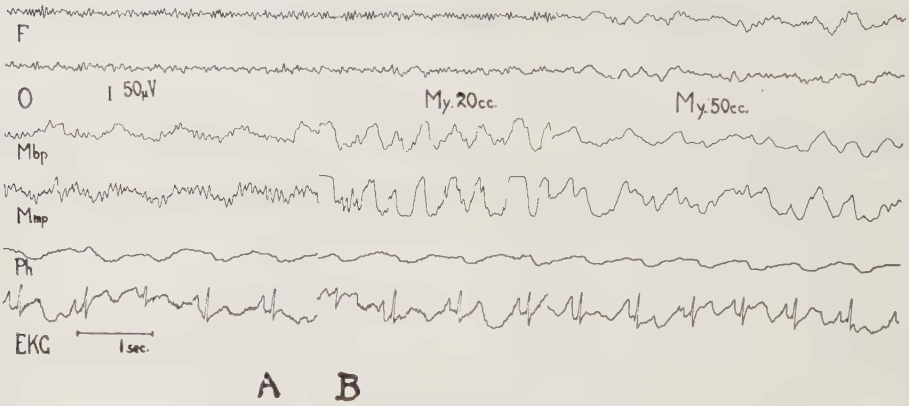

FIG. 3.

Effect of intravenous injection of 2% Tolserol solution (My) upon the tectum mesencephali in a case of severe pain. Mbp bipolar, Mmp monopolar records from the tectum of the midbrain. F bipolar frontal, O bipolar occipital records (scalp electrodes). A before, B after injection of the drug. Anesthesia: Atropine sulf. 0.6 mg; Nembutal 200 mg; Curare 100 units; Sod. Pentothal 0.375 g; N_2O-O_2.

upon the electrical discharges of the human cerebrum; the most pronounced changes were found in the electrical activity of the hypothalamus and of the midbrain.

We wish to express our appreciation to Dr. L. Krumperman and Dr. B. Siman from the Anesthesia Department for their kind cooperation.

Received October 14, 1949. P.S.E.B.M., 1949, **72**.

Further Studies on the Histochemical Specificity of Phosphatases.* (17464)

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In continuation of previous studies,[1] 10 more substrates were tried in histochemical experiments on phosphatases. The new substrates were the following: catechol- and hydroquinone monophosphates; glucose-6-phosphate, fructose-6-phosphate, phosphoglyceric acid; yeast adenylic acid (3-nucleotide), muscle adenylic acid (5-nucleotide); dimethyl- and diphenyl acid pyrophosphates, and casein. The first two were prepared by a modification of the King-Nicholson[2] procedure; instead of the Ba salts the Ca salts were prepared; the crude products were purified by extraction with water at an alkaline reaction and precipitation by 1 to 2 volumes of alcohol. Glucose-6-phosphate was the gift of Dr. B. Vennesland of the Dept. of Biochemistry, University of Chicago; fructose-6-phosphate, phosphoglyceric acid and yeast adenylic acid were obtained from the Schwarz Laboratories, New York; 5-nucleotide was purchased from the Sigma Chemical Co., St. Louis; dimethyl acid pyrophosphate was received from the Victor Chemical Works, Chicago; diphenyl acid pyrophosphate was synthesized according to the method of Neuberg and Wagner.[3]

Experimental. The technic was the same as reported previously. The substrates were used at two pH values only, at pH 5 and 9.2. Slides incubated with glycerophosphate were used as standards for comparison at both pH values. No reaction was obtained with casein in any of the tissues either at alkaline or at acid reaction.

In the alkaline range, intense reactions were observed in 2 hours with all other substrates, except the pyrophosphates which required prolonged incubation (up to 24 hours). The picture of distribution of enzymatic activity was essentially identical with all substrates, except 5-nucleotide. Minor and mostly inconstant differences were noted with the pyrophosphates, the nuclei being stained relatively more intensely than with the use of the other substrates.

Pictures obtained with the use of 5-nucleotide were different in several respects from those observed with any of the other substrates. Although in some of the organs (kidney, adrenal, intestinal mucosa, embryonic skeleton, placenta, etc.) the pattern of enzymatic distribution was indistinguishable from that seen with the use of, *e.g.*, glycerophosphate, in other organs patterns entirely different and specific for 5-nucleotide were found. Some of the main differences were as follows:

1. In the brain of the rat and mouse, nonspecific reaction is found mainly in the grey cortex where the reaction is rather diffuse, and in the walls of blood vessels (Fig. 1). With 5-nucleotide, the cortex is only slightly stained, and the reaction is mainly in the axons. Intense staining is obtained at some sites where the nonspecific reaction is practically negative such as the caudate nucleus (Fig. 2), the radiation of the corpus callosum and the

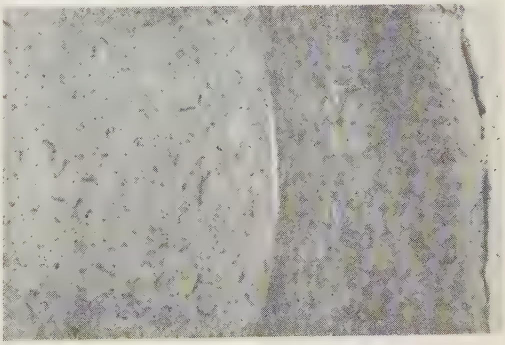

Fig. 1.
Brain of the mouse. Substrate: glycerophosphate. Grey cortex and blood vessels stained.

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* This work has been done under grants from the Douglas Smith Foundation for Medical Research of The University of Chicago, and from the Pathology Study Section of the U. S. Public Health Service.

[1] Gomori, G., Proc. Soc. Exp. Biol. and Med., 1949, **70**, 7.

[2] King, E. J., and Nicholson, T. F., *Biochem. J.*, 1939, **33**, 1182.

[3] Neuberg, C., and Wagner, J., *Biochem. Z.*, 1926, **171**, 485.

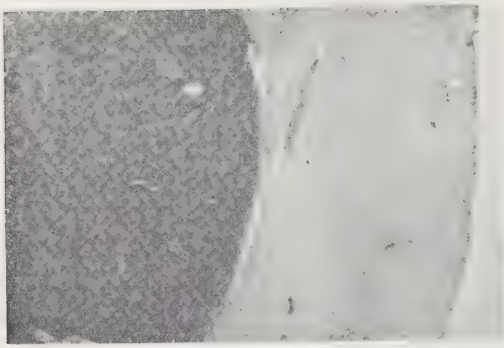

FIG. 2.
Another section of the same block. Substrate: 5-nucleotide. Caudate nucleus intensely stained.

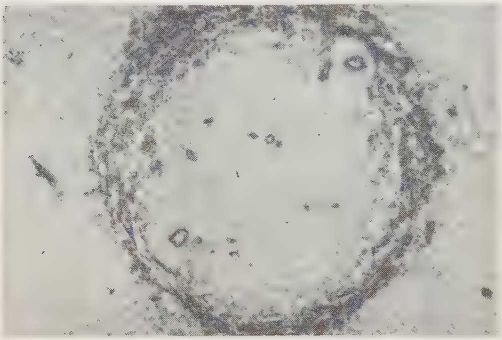

FIG. 3.
Human spleen. Substrate: glycerophosphate. Positive reaction at the periphery of a Malpighian body.

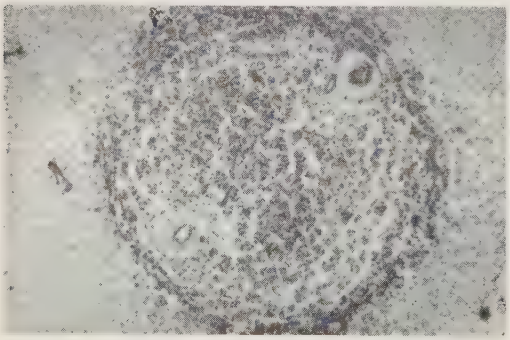

FIG. 4.
Consecutive serial section to the previous one. Substrate: 5-nucleotide. Reaction throughout the Malpighian body.

structures of the cornu Ammonis. The blood vessels are less intensely stained.

2. In the human spleen, only the periphery of the Malpighian bodies shows a nonspecific activity (Fig. 3); with 5-nucleotide, the centers are also intensely stained (Fig. 4).

3. In the testis of man, the mouse and the rat, 5-nucleotidase activity is quite intense in the nuclei of spermatogenic elements while nonspecific activity is much weaker.

4. Smooth muscle of vessel walls and of some viscera (urinary bladder, stomach, etc.) stains intensely although neither uniformly nor consistently when the substrate is 5-nucleotide, while the nonspecific reaction is negligible in the same locations. On the other hand, longitudinal muscle of the uterus of the mouse stains equally well with all substrates.

These findings seem to confirm previous contentions[4,5,6] that 5-nucleotidase is a specific enzyme, different from nonspecific alkaline phosphatase. Whether the multiple overlapping of patterns of distribution is genuine or in part due to impurities of substrate nature in the muscle adenylic acid used in these experiments will require further investigation.

In the acid range, good results were obtained with catechol monophosphate and with glucose- and fructose-6-phosphates; with the other substrates the reactions were much weaker and variable. No significant differences in localization of activity were seen with different substrates, except that phenylpyrophosphate tended to show a predominantly nuclear pattern of activity. The findings of Reis[7] according to which prostatic acid phosphatase is also primarily a 5-nucleotidase could not be confirmed histochemically since the intensity of the reaction was far more marked either with glycerophosphate or with 3-nucleotide than with 5-nucleotide as a substrate.

Summary. The pattern of distribution of alkaline phosphatase activity in tissues is, when 5-nucleotide is used as a substrate, different from patterns obtained with any other substrate. The specificity of 5-nucleotidase appears to be an established fact.

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[4] Reis, J., *Bull. soc. chim. biol.*, 1934, **16**, 385.

[5] Reis, J., *Enzymologia*, 1937-38, **2**, 110.

[6] Gulland, J. M., and Jackson, E. M., *Biochem. J.*, 1938, **32**, 590, 597.

[7] Reis, J., *Enzymologia*, 1938, **5**, 251.

Received October 20, 1949. P.S.E.B.M., 1949, **72**.

Absorption and Excretion of Streptomycin Para-aminosalicylate.* (17465)

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(Introduced by C. C. Sturgis)

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Streptomycin has become firmly established as a valuable agent in the treatment of selected cases of human tuberculosis. Para-aminosalicylic acid (hereafter designated PAS) is effective in the treatment of experimental tuberculosis in animals[1] and appears to enhance the effectiveness of streptomycin when it is used in combined therapy in experimental infections.[2] Recent evidence from clinical trials[3,4] suggests that a combination of parenteral streptomycin and oral PAS acts to delay the accumulation of strains of tubercle bacilli resistant to either drug.

Basic streptomycin can be combined with 3 molecules of PAS to form streptomycin para-aminosalicylate‡ (hereafter designated streptomycin PAS); one gram of streptomycin combining with 0.8 g of PAS. The resulting chemical is very slightly, if at all, more irritating when injected intramuscularly than is an equivalent amount of streptomycin. It has been found to be superior to streptomycin in experimental tuberculosis in the mouse;[5] a daily dose of streptomycin PAS, equivalent to 0.4 mg of streptomycin affording the same degree of protection as that produced by 2 to 3 mg of streptomycin sulphate. The present report concerns the results of investigation into the fate of streptomycin PAS in human subjects.

Materials and methods. The subjects were young patients with minimal or moderately advanced pulmonary tuberculosis as the only significant deviation from normality. Streptomycin PAS was injected intramuscularly in quantities equivalent to 1.0 g of streptomycin (0.8 g of PAS) in 4 subjects, and 0.5 g of streptomycin (0.4 g of PAS) in 4 other subjects. Plasma specimens (heparinized) were obtained for control purposes, and at 15 minutes, 30 minutes, and 1, 2, 4, 8 and 12 hours following injection of the material. Urine was collected in 3 pooled specimens each covering a 4 hour period. For purposes of comparison 2 of the above subjects were given an amount of PAS (0.8 g) orally equivalent to that contained in the streptomycin PAS which they had received by intramuscular injection in a previous experiment. Two subjects were given streptomycin PAS orally and in this case plasma specimens were collected only at 30, 60 and 120 minutes. Concentrations of PAS in plasma were determined by the method described by Marshall[6] using sodium para-aminosalicylate as standard material for calibration of the Evelyn colorimeter. Streptomycin concentrations were measured by the cup-plate method of bioassay in use in this laboratory. According to custom, concentrations of PAS are expressed as mg per 100 ml while concentrations of streptomycin are expressed as γ per ml. The control plasma in most individuals contains interfering substances which give readings for PAS estimated as near, but slightly less than 0.05 mg per 100 ml. This figure has, there-

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* This investigation was supported, in part, by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

† Charles Pfizer Fellow in Chemotherapy.

[1] Feldman, W. H., Karlson, A. G., and Hinshaw, H. C., *Proc. Staff Meet., Mayo Clin.*, 1947, **22**, 473.

[2] Youmans, G. P., Youmans, A. S., and Osborne, R. R., *Journal-Lancet*, 1947, **67**, 403.

[3] Karlson, A. G., Pfuetze, K. H., Carr, D. T., Feldman, W. H., and Hinshaw, H. C., *Proc. Staff Meet., Mayo Clin.*, 1949, **24**, 85.

[4] Delaude, A., Karlson, A. G., Carr, D. T., Feldman, W. H., and Pfuetze, K. H., *Proc. Staff Meet., Mayo Clin.*, 1949, **24**, 341.

‡ Prepared and furnished by the Charles Pfizer Company, Brooklyn, N. Y.

[5] Hobby, G. L., and Lenert, T. F., Presented at the 7th Streptomycin Conference, Veterans Administration, Denver, April 1949.

[6] Marshall, E. K., Jr., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 471.

fore, been accepted as a baseline. The addition of streptomycin PAS to water or serum gives recoveries, by the above methods, very near the expected values for both PAS and streptomycin. This would appear to indicate that the chemical union does not interfere with the biologic activity of streptomycin nor with the chemical determination of PAS.

Results and discussion. The plasma streptomycin concentrations, measured in the subjects who received streptomycin PAS intramuscularly, varied but were within the range expected for an equivalent amount of streptomycin. The values obtained for plasma PAS likewise varied considerably, particularly in the early minutes of absorption. Peak values developed between 15 and 30 minutes after intramuscular injection and averaged 1.35 mg per 100 ml in the subjects receiving an amount of streptomycin PAS equivalent to 1.0 g of streptomycin. In those subjects receiving a dose one-half this size the peak plasma concentrations averaged 0.6 mg per 100 ml. Concentrations fell rapidly and were at the base line after 4 hours in the group receiving the smaller dose and only very slightly above the base line in those who received the larger amount. This data is illustrated in Fig. 1. In Fig. 2, the curve of plasma PAS following ingestion of 0.8 g of PAS is compared with that obtained, in the same individual, following intramuscular administration of an equivalent amount of PAS as streptomycin PAS. The curves are very similar save that the peak concentration appeared later when the material was taken orally. Virtually the same relationships were observed in a second individual.

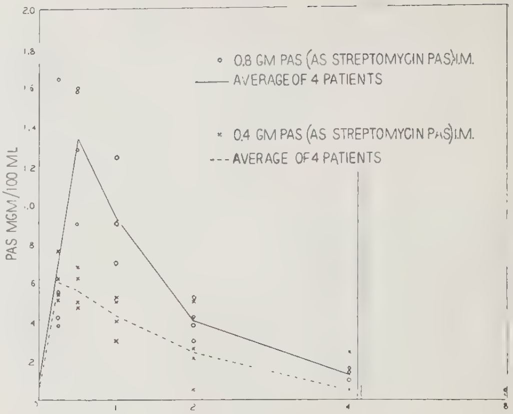

Fig. 1.

Plasma concentrations of PAS following intramuscular administration of streptomycin PAS in amounts equivalent to 1 g of streptomycin (0.8 g PAS) and 0.5 g streptomycin (0.4 g PAS). The lines indicate average values for each dosage level.

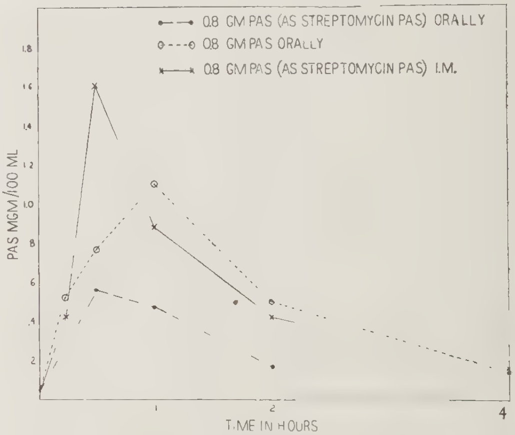

Fig. 2.

Comparison of plasma concentrations of PAS following equivalent amounts of: (1) Streptomycin PAS intramuscularly. (2) Streptomycin PAS orally. (3) PAS orally.

One fairly consistent feature of these experiments was the disassociation between the curves for PAS and for streptomycin concentrations in plasma. Peak levels for PAS appeared between 15 and 30 minutes while those for streptomycin occurred from 1 to 2 hours after injection (Fig. 3). Presumably this is due to rapid conjugation of the PAS radical[7] although it may be due, at least in part, to breakdown of the compound followed by independent behavior of streptomycin and PAS or its derivatives. Calculations of the expected levels of PAS, based on the concentrations of streptomycin in the plasma, were often fairly close to the observed levels of PAS for specimens taken 15 minutes after injection of the material. Later specimens usually exhibited PAS levels far below the calculated values, which might be expected if the PAS were undergoing rapid conjugation.

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[7] Way, E. L., Smith, P. K., Howie, D. L., Weiss, R., and Swanson, R., *J. Pharm. and Exp. Therap.*, 1948, **93**, 369.

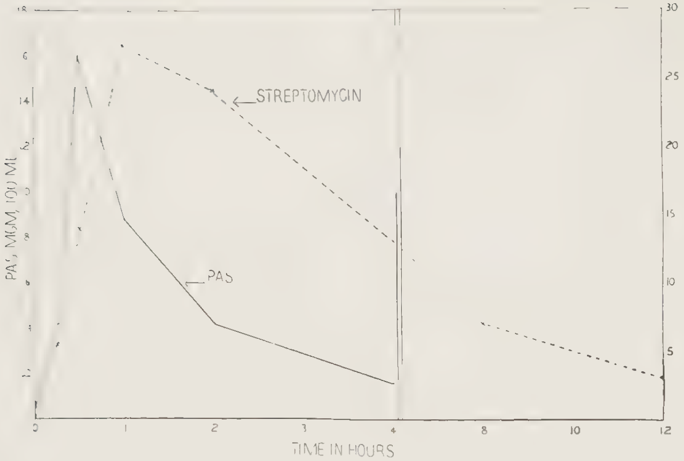

Fig. 3.

Plasma values for PAS and for streptomycin following intramuscular administration of an amount of streptomycin PAS equivalent to 1 g of streptomycin.

Streptomycin could not be detected in the plasma following oral administration of streptomycin PAS. The concentrations of PAS which developed were considerably lower than those expected from an equivalent amount of PAS, taken by mouth, or injected intramuscularly as streptomycin PAS, indicating that the material is certainly broken down to its components in the gastro-intestinal tract, though possibly not completely.

Urinary recovery of PAS following intramuscular administration of streptomycin PAS ranged from 9 to 24% (average 14%) with virtually the entire amount appearing within 4 hours of administration. Recovery of streptomycin averaged the expected 60%.

The concentrations of PAS in plasma following intramuscular administration of streptomycin PAS are not particularly impressive when compared with those following large amounts of PAS given orally (peak concentrations of 4 to 8 mg per 100 ml following doses of 3 g). They assume somewhat greater significance, however, when considered in relation to the sensitivity of newly isolated strains of tubercle bacilli which are reported[4] to be sensitive to PAS in concentrations in the neighborhood of 0.012 mg per 100 ml of medium. These results, coupled with the effectiveness of the material in experimental tuberculosis[5] and the simplicity of administration, would seem to indicate that streptomycin PAS is worthy of clinical trial in human tuberculosis on an experimental basis.

Summary. Streptomycin PAS may be given intramuscularly without undue irritation. Chemical assay of PAS, and bioassay of streptomycin, each give virtually quantitative recovery of streptomycin PAS *in vitro*. Following intramuscular administration of streptomycin PAS in human subjects, streptomycin was demonstrated in the plasma and urine within the expected range. PAS also appeared in significant concentrations, but the quantitative relation to streptomycin was altered.

Received October 17, 1949. P.S.E.B.M., 1949, **72**.

Liberation of Inorganic Phosphate from Adenosinetriphosphate by Fractions Derived from Developing Rat Muscle.* (17466)

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The systematic investigation of chemical changes occurring in differentiating muscle tissue was begun[1,2] by assaying quantitatively the main protein fractions at different stages of development. The direct continuation of this analysis was not immediately possible because the preparative characterization of the most significant muscle proteins, the myosin group, was impeded by the similarity in solubility of the nucleoproteins[3] and the discrepancy between the increase in quantity of the myosin proteins and the activity of adenosinetriphosphatase which usually are thought to be closely associated. As an explanation of this discrepancy (1) an appreciable increase in the enzymatic activity in fractions other than the myosin proteins, or (2) an increase in activity of the myosin fraction itself or (3) a simultaneous occurrence of both have been considered. These possibilities have been tested experimentally and the results are here described.

Experimental: ATPase activity determinations. About 500 mg of fresh, minced muscle tissue were homogenized in 10 cc of .5 M KCl in the cold. The pH was adjusted to 7.5 and the homogenate was divided into two 5 cc portions. One was stored in the cold for a determination on the whole (W). The other was subjected to protein fractionation as follows: the homogenate was stirred for one hour at 40°F, and then centrifuged for ½ hour at 3,000 r.p.m. The residue is R. The supernatant was carefully adjusted to pH 5.7 by adding split drops of .04 N HCl. The insoluble material was separated by centrifugation for ½ hour and denoted as M_1. The supernatant was diluted with 2 volumes of water, and then centrifuged for ½ hour. The residue is M_2. The supernatant is S. The fractions so separated were rehomogenized in 5 cc of .5 M KCl, and the pH was adjusted to 7.2-7.5. After dilution of each fraction and the whole, so that the activity would fall within a measurable range, .2 cc portions were taken for ATPase activity determinations, which were performed by the procedure of Dubois and Potter.[4] The ATP was prepared according to the method of Dounce, *et al.*[5]

Tubes set up for phosphate determination contained the following: .60 cc of 2.5% ammonium molybdate in 5 N H_2SO_4, .25 cc of sample, distilled water to dilute to 3.00 cc, and lastly, 15 cc of .25% 1-amino, 2-naphthol, 4-sulfonic acid. Phosphate standards and a blank were run concomitantly. The contents were thoroughly mixed and heated in a water bath at 37°C for 5 minutes. After cooling the samples to room temperature, colorimetric readings were taken on the Beckman spectrophotometer at 660 mμ.

Nitrogen determinations for each fraction in suspension were made by the Kjeldahl method modified by Boell[6] after precipitation with trichloracetic acid (100 g to 100 cc) to yield a solution concentration of 5%.

Results and discussion: The enzymatic liberation of inorganic phosphate from adenosine triphosphate by 4 protein fractions obtained from mesoderm or muscle tissue at different stages of development is shown in Table I. The activities are computed as phosphate liberated *either* per mg of N in the

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* Supported in part by a grant from the American Cancer Society through the NRC Committee on Growth.

[1] Herrmann, Heinz, and Nicholas, J. S., *J. Exp. Zool.*, 1948, **107**, 177.

[2] Herrmann, Heinz, and Nicholas, J. S., *J. Exp. Zool.*, 1948, **107**, 165.

[3] Herrmann, Heinz, and Nicholas, J. S., *J. Exp. Zool.*, 1949, **112**, 253.

[4] Dubois, K. P., and Potter, V. R., *J. Biol. Chem.*, 1943, **150**, 185.

[5] Dounce, A. L., Rothstein, A., Beyer, G. Thannhauser, Meier, R., and Freer, R. M., *J. Biol. Chem.*, 1948, **174**, 361.

[6] Boell, E. J., *Trans. Conn. Acad. Arts and Sci.*, 1945, **36**, 429.

fraction (fractional activity) or per mg of N in the whole homogenate (relative activity). The first way of computing indicates the changes of activity which occur during the development within the respective fraction, independent of the changes of the quantity of this fraction. The second method of computation provides an index for the contribution of enzymatic activity of one particular fraction relative to the enzymatic activity of the whole homogenate of muscle tissue. If the enzymatic activities for the single fractions computed for the total nitrogen are added, the activity obtained theoretically should be equal to the activity of the whole unfractionated tissue homogenate. The latter data correspond to the figures represented in the graph in the previous paper.[1] The figures obtained by computation for the total nitrogen, (relative activities) are dependent not only upon the activity in the respective fraction but also upon the quantity of this fraction relative to the other fractions composing the total homogenate. In order to show the scattering of our results we have listed in Table I the numbers of samples used and the range for the fractional activities and also the averages for single fractions and for the whole homogenate as well as the relative activities for the single fraction and for the sum of the fractions to be compared with the activity of the whole unfractioned tissue. From these figures it is quite evident that all fractions show a marked increase in activity. It can be noticed that in the 3 fractions, R, M_1, M_2, almost the entire increment is found for the development from the 14th to the 19th day of gestation, the period in which the most pronounced steps of structural and functional differentiation are taking place.

Fraction S increases at a steadier rate over the whole developmental period investigated. The increase in the relative activities (calculated per mg nitrogen of the total homogenate) differs from the fractional activities by its dependence upon the quantity of the respective fraction. A closer correlation of the two sets of figures was not attempted. However it can be clearly recognized that in the case of fractions R, M_2, and S the increases in the relative activities are of the same order of

TABLE I. Activities in μg Phosphorus Liberated in 15 Min. at 37.5°C per mg N.

Fraction	14 days gestation			19 days gestation			1-2 days after birth			9-10 days after birth		
	No. rats	Range	Avg	No. rats	Range	Avg	No. rats	Range	Avg	No. rats	Range	Avg
				Activity of individual fraction								
R	2	66-80	73	2	480-500	490	3	720-1000	850	3	610-720	670
M_1	3	55-140	91	2	550-680	615	4	720(1400)*-1700	1300(1500)	3	860-1300	1050
M_2	3	50-88	70	2	82-88	85	4	110(560)-870	530(670)	4	420-850	570
S	4	170-300	220	2	410-420	415	4	460(630)-850	660(720)	3	720-1100	920
W	2	120-130	120	2	400-890	645	4	830-980	910	4	800-1300	970
				Activity calculated per mg N of whole homogenate								
R		47			200			360			300	
M_1		5			54			180(190)			280	
M_2		15			40			48(62)			50	
S		*66*			*250*			*170(190)*			*220*	
Σ		133			544			758(802)			850	
W		120			490			910			970	
(W)		95			570			570			950	

Σ = The sum of activities of single fractions per mg N of the whole homogenate.
(W) = Values recalculated from previous data for activities of the whole homogenate corrected for increase in activity in the presence of KCl.
* The wide spread is due to one determination which is out of line with the others. If this experiment were discarded the spread would be as indicated by the figures in parentheses.

magnitude as the increases in fractional activities. In contrast to these fractions an exceptionally great increase in the relative activity is found in the M_1 fraction. In this instance an increase of 14 times for the fractional activity is to be compared with an increase of about 56 times for the relative activity. This difference is due to the rapid increase in the quantity of the M_1 fraction which has been described before.

The values for the relative activities in Table I, when added (R, M_1, M_2, S) should equal the activities found in the whole homogenate (W). Since the two sets of figures are in close agreement, it can be concluded that the values secured for the relative activities give a correct measure for the proportion in which these individual fractions contribute to the activity of the whole homogenate. The increase in activity for the whole homogenate found in this series corresponds closely to the values obtained previously.[3] The present values are consistently somewhat higher than the previous ones since the activity of the whole when homogenized in KCl is 18% higher than when homogenized in water, due to activation by the K ion. For the first day after birth the earlier value (W), which is more than 35% lower than the recent value W, forms a "dip" in the curve in previous experiments. Since the rate of increase in activity is greatest up to the time of birth, the present higher value, W, is the more reasonable one. The difference between the rapid increase in enzymatic activity and the slow increase in the quantity of the total fraction of myosin proteins (the sum of the present fractions $M_1 + M_2$) which appeared previously as a discrepancy can now be explained. The former assumption was that most of the apyrase activity in muscle at all stages of development should be linked to the myosin protein fraction and should remain fairly constant for the unit of this fraction. It now appears that only a minor part of the total activity is contributed by the myosin proteins; although a large increase in activity does occur within the myosin fraction itself.

The problem of the nature of the proteins of the myosin group as well as the relationship of the different enzymes with apyrase activity is at present in a state of flux. As to the former, discussion centers around Szent-Gyorgyi's[7] separation of the myosin complex into actin and myosin, and recently Bailey seems to have found evidence for a new member of the myosin family (his tropomyosin). Enzymes with apyrase activity and distinctly different properties recently have been described from various sources[8-10] and even in muscle a distinction of ATPase enzymes was recently claimed. The fact that there is an increase during embryonic differentiation of all fractions of muscle tissue is in agreement with the observations of Potter,[11] Flexner[12] Barth,[13,14] and Moog[15] who find an increase in apyrase enzymes during differentiation in a great variety of mammalian and even invertebrate tissues. In these instances the increase means a more or less indirect role of apyrases in the elaboration and maintenance of specific structures and functions. In the M_1 fraction this relation is a more direct one since the development of enzymatic and structural properties of the myosin proteins in muscle represents directly the products of differentiation in a highly specialized tissue.

Summary. In a quantitative chemical study of developing muscle there is a greater activity of adenosinetriphosphatase than is warranted by the increase in amount of myosin proteins. In order to ascertain the reason for this discrepancy determinations of phosphatase activity were made on fractions of devel-

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[7] Szent-Gyorgyi, A., Chemistry of Muscular Contraction, Academic Press, Inc., New York, 1947, 150 pp.

[8] Barth, L. G., and Jaeger, L., *J. Cell. and Comp. Physiol.*, 1947, **30**, 111.

[9] Kielley, W. W., and Meyerhof, O., *J. Biol. Chem.*, 1948, **174**, 387.

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[13] Barth, L. G., *J. Trans. N. Y. Acad. Sci.*, 1949, Ser. II, Vol. II, 108.

[14] Jaeger, L., and Barth, L. G., *J. Cell. and Comp. Physiol.*, 1948, **32**, 319.

[15] Moog, F., *J. Exp. Zool.*, 1947, **105**, 209.

oping muscle obtained from rat embryos of 14 days, fetuses of 19 days, rats of 1-2 days and 9-10 days of age. The activity was calculated in micrograms of phosphorus liberated in 15 min. at 37.5° per mg N.

The activity was determined for each of 4 fractions and the sum of these, the activity of the whole homogenate, was in close agreement with previous determinations of the whole homogenates.

All 4 fractions increase in activity during development; the M_1 fraction, containing the purest preparation of the myosin proteins develops in activity 6 times faster than the other fractions.

While the apyrase activity was formerly thought to be limited to the myosin protein fraction this contributes but a minor part of the total activity.

Received August 8, 1949. P.S.E.B.M., 1949, **72.**

Role of Pyridoxine in the Production of Leucocytes in Normal and Leukemic Mice.* (17467)

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Morgan, Groody, and Axelrod[1] showed that pyridoxine-deficient dogs developed a leucocytosis with an absolute increase in the numbers of circulating neutrophiles and an absolute decrease in the numbers of lymphocytes. These abnormalities could be corrected by giving adequate doses of pyridoxine. In the same year McCall, Waisman, Elvehjem, and Jones[2] described similar leucocyte changes in pyridoxine-deficient and in pantothenic acid-deficient monkeys. They stated that the reversal of the polymorphonuclear to lymphocyte ratio also occurred in riboflavin deficiency. In the pyridoxine-deficient animals the reversal could not be corrected completely by pyridoxine but could be corrected by whole liver. Wintrobe and his associates[3] observed a tendency of the granulocytes to increase in pyridoxine-deficient swine, but there was no reversal of the ratio of polymorphonuclears to lymphocytes. Stoerk, Eisen, and John[4] described thymus atrophy and a reduction in the number of fixed and circulating lymphocytes, and Stoerk[5] described lymphoid atrophy and regression of rat lymphosarcoma in pyridoxine-deficient rats. In order to facilitate the production of pyridoxine deficiency, Ott[6] investigated the antipyridoxine activity of desoxypyridoxine hydrochloride (2,4-dimethyl-3-hydroxy-5-hydroxymethylpyridine). He found that two molecules of the analogue were sufficient to offset the vitamin activity of one molecule of pyridoxine, as measured by chick growth. Under other conditions[7] much larger quantities of the analogue are necessary. These observations prompted us to investigate the effect of both pyridoxine deficiency and pyridoxine excess on the circulating leucocytes and hematopoietic organs of normal mice and of mice having various abnormalities of the leucocyte pattern.

Pyridoxine Deficiency in Normal Mice.

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* This research was made possible by grants from the National Vitamin Foundation and the Price McKinney Memorial Fund.

[1] Morgan, A. F., Groody, M., and Axelrod, H. E., *Am. J. Physiol.*, 1946, **146**, 723.

[2] McCall, K. B., Waisman, H. A., Elvehjem, C. A., and Jones, E. S., *J. Nutrition*, 1946, **31**, 685.

[3] Wintrobe, M. W., Follis, R. H., Miller, M. H., Stein, H. J., Alcayaga, R., Humphreys, S., Suksta, A., and Cartwright, G. E., *Johns Hopkins Hosp. Bull.*, 1943, **72**, 1.

[4] Stoerk, H. C., Eisen, H. N., and John, H. M., *J. Exp. Med.*, 1947, **85**, 365.

[5] Stoerk, H. C., *Fed. Proc.*, 1948, **7**, 281.

[6] Ott, W. H., Proc. Soc. Exp. Biol. and Med., 1946, **61**, 125.

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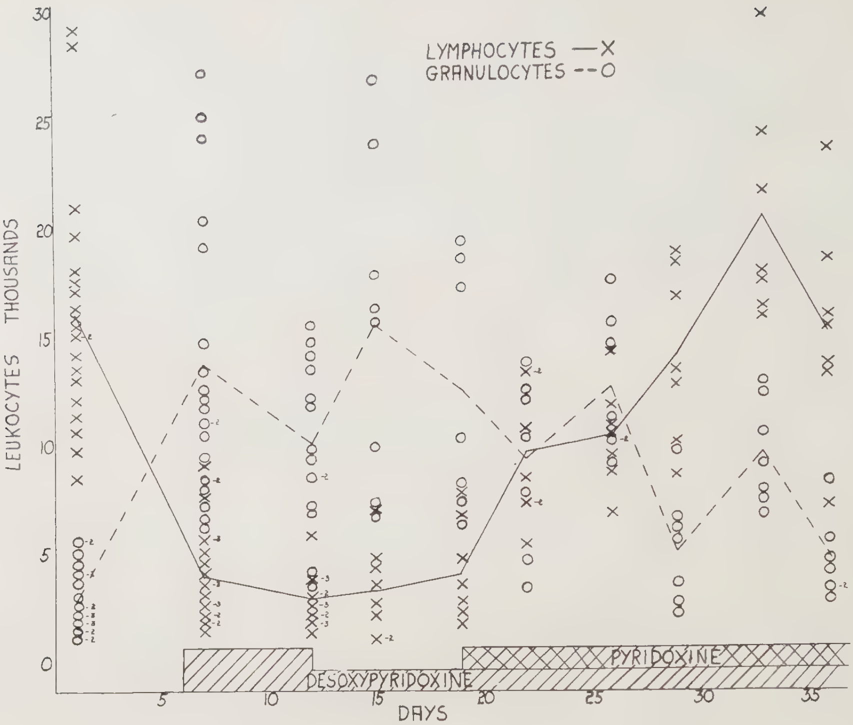

FIG. 1.
Leucocyte changes in pyridoxine-deficient normal mice.

Weanling mice of a Swiss strain were placed in cages with wire-mesh bottoms. They were fed *ad libitum* a modification of a pteroylglutamic acid-deficient diet which has been described previously;[8] pyridoxine and succinylsulfathiazole were not included. The animals were given pteroylglutamic acid intraperitoneally in daily doses of 20 μg. In addition desoxypyridoxine[†] was given subcutaneously as a 2% solution of the hydrochloride. In a preliminary experiment, daily doses in excess of 1 mg were fatal within ten days. On a 1 mg dose the mean survival time was 20 days and weight loss was marked. Many of the animals developed the expected granulocytosis and lymphopenia but several did not. In a second experiment the animals were given 2 mg daily for a week and then 1 mg daily. Again the leucocyte response was variable and weight loss occurred rapidly. Also the leucocyte changes could not be corrected completely by the administration of pyridoxine. These unsatisfactory results using a purified diet suggested a trial of a crude diet (Purina dog chow) supplemented by larger amounts of desoxypyridoxine given parenterally, as a possible means of producing the desired alteration in the leucocyte pattern. This approach proved successful. Twenty Swiss mice were fed Purina dog chow *ad libitum*. On the 6th day subcutaneous injections of desoxypyridoxine HCl in a 2% solution were started. From the 6th to the 12th day 8 mg were given daily in 2 doses of 4 mg each at an interval of 8 hours. Because of a high mortality in the animals the doses were then halved to supply 4 mg daily, and this was continued to the end of the experiment; during this period only one mouse died. Supplementary pyridoxine HCl (0.2%) was added to the ground diet on the 19th day. Since the mice ate from 3 to 6 g of dog chow daily, they were supplied with 6 to 12 mg of supplementary vitamin daily. Smaller amounts used in preliminary experiments had failed to restore the leucocyte pattern to normal. Total and differential leucocyte counts were done twice weekly. At autopsy, sections of liver, spleen, marrow, thymus, lymph nodes, and both adrenals were taken for histologic study.

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[8] Weir, D. R., Heinle, R. W., and Welch, A. D., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 211.

[†] Kindly furnished by Merck & Co., Inc.

As shown in Fig. 1, a complete reversal of the ratio of granulocytes to leucocytes occurred very promptly after desoxypyridoxine was started, with development of absolute granulocytosis and lymphopenia. Within 3 days after the pyridoxine supplement was started restoration of the normal leucocyte pattern had begun, and was complete 14 days later (36th day). After the reduction of the dose of desoxypyridoxine during the initial period, only one animal died and the others remained in apparent good health. There was no significant weight loss, and they did not develop ruffled fur, convulsions, or obvious neurologic disturbances. Red blood cell counts were not done in this experiment, but, in a preliminary experiment which was conducted under identical conditions except for a lower dose of supplementary pyridoxine, the mean erythrocyte count did not change significantly.

At autopsy on the 36th day no gross or microscopic abnormality could be detected in the tissues. In order to obtain material for histologic study at a time when the reversal of the granulocyte to lymphocyte ratio was established, 20 mice were maintained on the regimen described above, but were not given the supplementary pyridoxine. Fifteen of the 20 survived to be killed on the 37th day, at which time granulocytosis and lymphopenia, with a complete reversal of the ratio, had been present for 32 days. None of these animals showed any evidence of lymphoid atrophy. The thymus glands and lymph nodes were normal in appearance, and the splenic follicles were, if anything, slightly hyperactive. There was no increase in the size of the adrenal glands or demonstrable change in their cytological structure. The marrows showed possible increase in cellularity due to increased numbers of granulocytes without increase in immature cells, although this change was not marked. The liver of one animal that died early showed extensive hemorrhagic necrosis. The liver of another that survived to be killed showed a few small foci of necrosis. There is reason to believe that a generalized tissue deficiency of pyridoxine had not been established. Growth was not impaired, convulsions did not occur, there was very slight anemia, and lymphoid regression or atrophy was not present at autopsy. It is likely that a partial deficiency is sufficient to produce the leucocyte shift without producing other demonstrable changes.

Effect of Pyridoxine Deficiency on the Leukemia of Ak Mice. In mice of the Ak strain the intravenous injection of cells from diseased older animals produces leukemia in 100% of younger animals.[9] In the animals now being bred in this laboratory this is believed to be a mixed type of leukemia, in which both adult granulocytes and adult lymphocytes participate, with the appearance of small numbers of immature blastic cells. About 10 to 15 days after transplanting, the total leucocyte count begins to rise. It remains elevated for 10 to 20 days and then falls toward normal levels shortly before the animal dies. Death usually occurs 25 to 35 days after the transplant. Differential counts show that the initial rise is due to an increase of granulocytes. The ratio of these cells to lymphocytes may be as great as 4 to 1. As the leukemia progresses the lymphocytes increase and, coincidentally with this rise, the granulocytes begin to fall and reach normal levels when the lymphocytes are still elevated. At death any elevation of counts above normal is due to increased numbers of lymphocytes. The immature blastic cells appear in small numbers at the height of the leucocytosis and persist until death. The effect of pyridoxine deficiency on this leucocyte pattern was investigated. Fourteen weanling Ak mice were placed on a diet of Purina dog chow. On the first day all animals were given intravenous injections of a suspension of cells from lymph node and spleen of a diseased older animal. On the 8th day a group of 9 was started on desoxypyridoxine hydrochloride. Each was given 1.5 mg subcutaneously twice daily. Total and differential leucocyte counts were done at the end of the first week and twice weekly

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[9] Weir, D. R., and Heinle, R. W., Proc. Soc. Exp. Biol. and Med., 1947, **66**, 268.

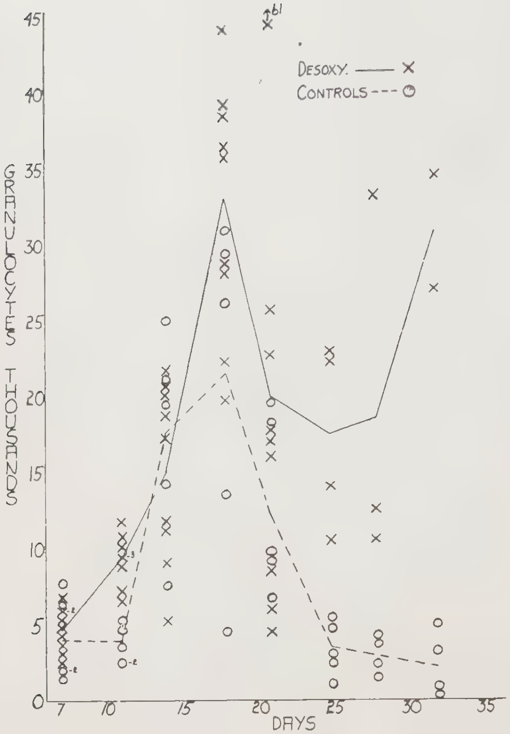

FIG. 2.
The effect of pyridoxine deficiency on the granulocytosis of Ak mouse leukemia.

thereafter. The mean survival time of the animals receiving desoxypyridoxine was 24.6 days as compared to 31.8 days for the controls. This decrease in survival time is statistically significant. The value for P is less than 0.02.[10] The leucocyte patterns are shown in Fig. 2. In the early stages of the leukemia the granulocyte counts of the experimental animals were generally higher than those of the controls. On the 25th day and thereafter all the granulocyte counts of the experimental animals were 2 to 6 times as great as the highest count shown by the controls during the same period. The lymphocyte counts of the experimental animals were generally lower than those of the controls but not significantly so. Histologic study of sections of liver, spleen, bone marrow and lymph nodes taken at autopsy failed to reveal any difference between the experimental and control groups.

Preliminary Studies of the Effects of Pyridoxine in Excess. Groups of normal mice were given parenteral pyridoxine HCl in divided daily doses of 8 and 16 mg for a period of 48 days. None of the animals showed any significant change in the leucocyte pattern. Three groups of 5 Ak mice were injected intravenously with a leukemic transplant. All were maintained on dog chow diet. One group received 2 mg of parenteral pyridoxine HCl in a single daily dose; another group received 4 mg; and the third group served as controls. The pyridoxine supplement failed to influence the course of the leukemia or to alter the histologic findings at autopsy. During the study of a myeloid metaplasia factor from human urine Heinle, Hirschmann, and Wearn[11] observed that the injection of certain extracts of urine from patients with chronic myeloid leukemia, produced a moderate to marked granulocytosis in mice. The effects of excess amounts of pyridoxine on this granulocytosis was studied. Ten adult Swiss mice were given for 10 days daily injections of a urine extract known to be potent. Five of these also were given pyridoxine hydrochloride parenterally in doses of 8 mg twice daily. The mean polymorphonuclear count of the 10 animals at the beginning of the experiment was 3370 per cu mm. At the end of the 10 day injection period the mean for the group receiving pyridoxine was 32,615; for those not receiving pyridoxine it was 43,544. The difference is not statistically significant.

Discussion. The results indicate that a deficiency of pyridoxine causes an increase in granulocyte production in normal mice and in mice dying from transplanted leukemia. It is known that vitamins of the B_6 group take part in transamination and decarboxylation,[12–14] and that the active compound is a phosphorylated form of pyridoxal acting as cotransaminase and co-decarboxylase.[15-18] In the intact normal animal the various compounds of the group presumably are freely interconvertible.[19,20] From these considerations, the hypothesis is suggested that the granulocytosis in pyridoxine-deficient mice is due to the failure on the part of the animals to transaminate, decarboxylate, or otherwise modify unneeded supplies of granulocyte-building substances, and that the animals thereupon utilize these unneeded supplies in producing excess numbers of granulocytes. It is not necessary to assume that the reverse would be true. A further hypothesis may be made that the granulocytosis of a disease state such as human myeloid leukemia may be due to a defect in the absorption or utilization of vitamins of the B_6 group. Although freely interconvertible in the intact animal, this may not be true of them in the diseased animal. Further investigations in mice and in leukemic human beings are in progress to test the validity of these hypotheses. It has been demonstrated that adrenocorticotropic and adrenal cortical hormones will cause granulocytosis and lymphopenia in animals.[21,22] In our histologic material we could not demonstrate evidence of adrenal cortical hyperplasia.

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[10] Fischer, R. A., Statistical Methods for Research Workers, Oliver and Boyd, Edinburgh, 1934.

[11] Heinle, R. W., Hirschmann, H., and Wearn, J. T., Approaches to Tumor Chemotherapy, 1947, 77.

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Summary. 1) Pyridoxine deficiency in mice produces granulocytosis and lymphopenia. 2). The leukemia of the Ak mice now being bred in this laboratory is participated in by both granulocytes and lymphocytes. In pyridoxine deficiency the leukemic granulocytosis is more severe and the survival time of the animals is decreased. 3) The hypothesis is advanced that the granulocytosis in pyridoxine-deficient mice is due to the failure on the part of the animals to perform metabolic functions in which pyridoxine derivatives play a catalytic role; thus, supplies of compounds used in the formation of granulocytes accumulate, and, as a result, the animals utilize this plethora in producing excess numbers of granulocytes. The implications of this hypothesis are discussed.

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Received August 10, 1949. P.S.E.B.M., 1949, **72**.

Device for Measuring Blood Pressure in the Unanesthetized Rat. (17468)

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The simple technic for measuring blood pressure in the rat described here consists of placing a cuff permanently around the abdominal arorta and vena cava just above their bifurcations and measuring the blood pressure by means of an oscillometer and mercury manometer.

Description of the Apparatus and Its Use. The aortic cuff is shown in Fig. 1. It consists of a latex bulb with a short stem which is fitted on to a piece of plastic tubing, and a plastic shell into which the bulb is inserted.

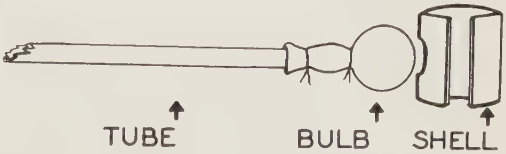

Fig. 1.
The aortic cuff unassembled.

To make the bulb a glass bead 4 mm in diameter with a stem attached is dipped into liquid latex. After drying for 24 hours, the latex is vulcanized by exposure to sulfur chloride fumes for one minute, and then washed under running tap water. After drying, the bulb is powdered and peeled off the mold. The short stem of the bulb is fitted into the end of a 12 cm piece of 18-gauge polyethylene tubing. The tubing is reinforced where it is fitted into the capsule by a 4 mm length of 18-gauge stainless steel tubing. The shell of the cuff consists of a plastic tube (Tenite II) 6 mm long with an inner diameter of 4 mm and an outer diameter of 6 mm. The shell is split lengthwise so that it can be slipped around the aorta. A 3 mm hole is drilled in the shell just opposite the slit and midway between the two ends. The bulb is fitted into the shell, with the tubing brought out through the hole. The bulb should just fill the inside of the shell. The cuff is washed in hot running tap water for 4 hours and then stored in 70% alcohol. Before use, each cuff is tested to withstand an air pressure of 300 mm Hg.

To place the cuff on the aorta the animal is anesthetized with ether and a midline incision is made in the abdomen. The aorta and vena cava are dissected free from the surrounding tissue, but are not separated from each other. Isolating the aorta was found to be difficult because of the danger of tearing the vena cava in the operation. The cuff is placed around both vessels just above their bifurcations. The shell is tightly closed with a strong ligature and secured firmly to the surrounding tissues. The tubing is brought through the posterior wall of the abdominal cavity and carried under the skin to the back of the neck where it is brought to the outside. In this position the tube cannot be disturbed by the animal (Fig. 2). Special care must be taken to prevent moisture from accumulating in the tube or bulb which could falsify pressure readings. There is no evidence that the animal suffers any inconvenience from the cuff. Autopsy examination of the aorta with the cuff *in situ* made 2 to 3 months after inserting the cuff has revealed very little fibrous tissue formation.

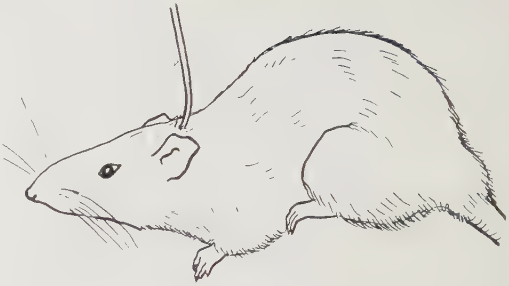

FIG. 2.
Rat showing place of exit for the plastic tubing leading from cuff.

Blood pressure readings are made with the use of an improvised oscillometer and a mercury manometer. This apparatus is shown in Fig. 3. The animal is placed in a metal holder (L) or in a 500 cc beaker. There is no struggling since the animal is not uncomfortable or irritated in any way. The tube from the cuff is connected with an oscillometer which consists of a 15 mm length of 1 mm capillary tubing (K) connected with a pressure bottle (G). The capillary tube contains a small drop of colored alcohol which serves as an indicator of the oscillations which are transmitted from the aorta. The pressure bottle G has a mercury manometer attached and it in turn is connected with a bottle of compressed air (A). The cuff is inflated through the capillary oscillometer with air supplied at a steady smooth flow through a hypodermic needle (F) from the reservoir A which has been adjusted to deliver pressures from 0 to 300 mm Hg in about 30 seconds. As the pressure rises in the cuff and oscillometer, the alcohol bead travels toward the cuff and shows distinct oscillations which reach a maximum intensity, gradually decrease, then abruptly diminish. The point of diminution is taken as the endpoint. A manometer reading is made at this point. A small pulsation persists after the endpoint. The pressure in the cuff is quickly released through the side tube H. Three to 5 readings are made at one-minute intervals.

Experimental data. This method for measuring blood pressure in the unanesthetized rat has been tested on a group of 20 normal male rats of the Sprague-Dawley strain which weighed from 105 to 165 g at the beginning of the study. Cuffs were placed around the

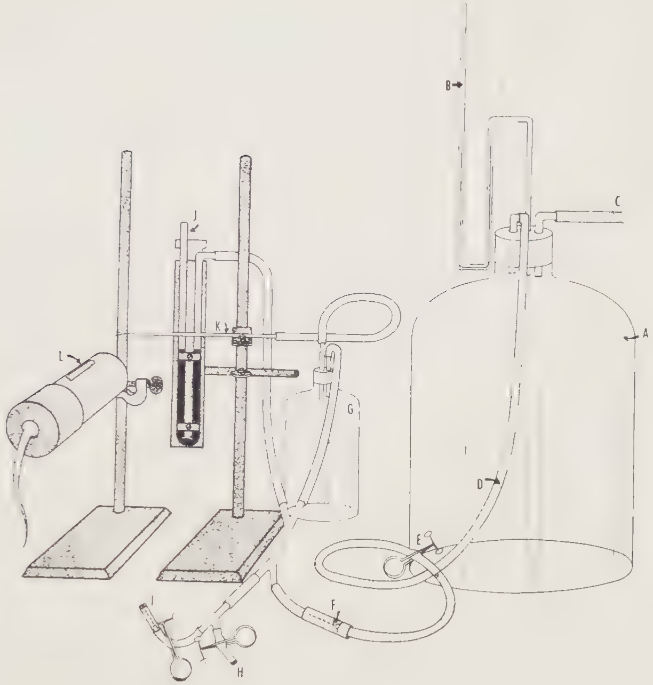

Fig. 3.
Oscillometer and manometer hooked up with pressure bottles. The oscillometer is connected to the tubing from the animal.

aorta and vena cava a few days before the first readings were made. Afterwards the blood pressure measurements were determined 3 times a week or oftener, over a period of 8 weeks. These readings are shown in Table I. The average of 540 blood pressure readings taken on the 20 normal unanesthetized rats over a 2 month period was 130 mm Hg.

The readings obtained by the cuff method were compared with the systolic pressure in the femoral artery measured intra-arterially by means of a capacitance manometer designed by Noble.[1] These readings were made on rats anesthetized with ether. Immediately after the intra-arterial reading, while the animal was still asleep, blood pressure readings by the cuff method were made. Twenty-nine rats were used for this study. Ten of this group had been made hypertensive by enclosing the left kidney with a latex capsule and removing the right kidney (Abrams and Sobin[2]). The remaining 19 rats were the normal animals which had been used previously for the blood pressure readings recorded in Table I. The comparative data are presented in graph form in Fig. 4. The intra-arterial systolic pressure for a given rat is plotted as the ordinate and the pressure reading by the cuff method as the abscissa. The diagonal line represents perfect agreement. The readings by the 2 methods in both normal and hypertensive animals tend to fall along this line. The average intra-arterial systolic pressure for the 19 normal animals was 121 ± 15 mm Hg and the reading by the cuff method while the animals were still under ether was 118 ± 15 mm Hg. Because of the close correlation of the readings by the 2 methods it is assumed that the readings by the cuff method indicate the approximate systolic blood pressures. It has been noted, however, that when the cuff is inflated to the "systolic pressure" reading the aorta may not be com-

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[1] Noble, F. W., Third Conference on Factors Regulating Blood Pressure, May 5-6, 1949, Josiah Macy, Jr. Foundation, New York.

[2] Abrams, M., and Sobin, S., Proc. Soc. Exp. Biol. and Med., 1947, **64**, 412.

TABLE I.
Systolic Blood Pressures as Determined by the Oscillometric Method on a Group of 20 Adult Male Rats Over a 2-Month Period.

Rat No.	Blood pressure, mm Hg weekly avg								Avg for 8-wk period
	1st	2nd	3rd	4th	5th	6th	7th	8th	
760	123	129	119	119	117	120	122	121	121
761	125	123	115	120	116	124	124	132	122
762	137	121	124	123	137	137	127	142	131
763	139	134	134	143	136	126	120	133	133
764	140	119	123	128	117	129	129	128	127
765	134	126	133	123	117	131	142	138	131
767	139	126	124	126	111	124	121	126	125
768	128	110	123	129	120	134	141	143	129
769	138	108	132	118	119	130	148	163	134
770	136	129	125	126	113	133	131	133	128
771	141	118	133	127	133	141	135	135	133
772	126	126	115	126	130	130	135	135	128
773	124	116	130	120	120	129	130	133	125
774	121	124	125	137	127	133	132	137	130
775	—	142	130	122	146	134	149	138	137
776	136	133	140	145	137	155	120	—	138
777	120	129	130	131	129	135	—	—	129
778	131	141	145	160	138	—	—	—	143
779	—	124	128	129	141	125	136	133	131
781	—	133	126	113	118	120	122	123	120
Weekly group avg	131	126	128	128	126	131	131	135	

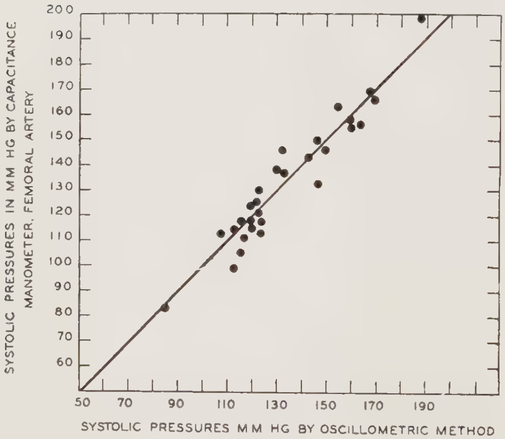

FIG. 4.

Comparison of the intra-arterial pressure by the capacitance manometer with the indirect pressure by the oscillometric method.

pletely occluded, for pulsations are still recorded by the capacitance manometer.

Simultaneous occlusion of the thoracic aorta and inferior vena cava has been shown to increase blood pressure in the dog (Barcroft[3]). However, in our observations no measurable change occurred in the intracarotid pressure when taken simultaneously with the pressure readings made with the cuff on the abdominal aorta and vena cava.

Summary. A device for measuring blood pressure in the aorta of the unanesthetized rat is described. It is simple and permits an unlimited number of readings over a long observation period.

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[3] Barcroft, H., *J. Physiol.*, 1931, **71**, 280.

Received Sept. 9, 1949. P.S.E.B.M., 1949, **72**.

Precipitation of Insulin by Lysozyme and Effect of the Complex on the Blood Sugar Level. (17469)

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Because of the possible therapeutic application of a lysozyme-insulin complex, the properties of which might be expected to resemble those of protamine-insulin, experiments have been done on the precipitation of insulin by crystalline egg lysozyme (Armour) isoelectric point 10.5 to 11[1,2] and lysozyme which was treated in several different ways intended to destroy its lytic power as tested on *M. lysodeikticus*. The effect of the resuspended precipitates on the blood-sugar level of rabbits was also tested.

Table I shows the data obtained in measuring the precipitation of zinc-containing crystalline insulin dissolved in a minimal volume of N/100 HCl, adjusted to pH 7.3 with N/100 NaOH and made up to 0.10% concentration. The indicated quantities of a 0.10% (uncorrected for 9.56% moisture) solution of lysozyme were added to 1 ml of the insulin solution and the volume made up to 2 ml with water. After standing over night the tubes were centrifuged and the supernatant fluids tested for insulin and lysozyme by adding more of one or the other reagent. The precipitates were washed twice with 2 ml of water, centrifuging both times, and analyzed for nitrogen by the Kjeldahl method using H_2SO_4 and H_2O_2 for digestion, the color being developed with Nessler's reagent and measured in a photoelectric colorimeter. All the mixtures were set up and analyzed in duplicate.

While 0.60 ml of a 0.10% solution of untreated lysozyme precipitated almost all the insulin in 1 ml of the insulin solution,* 96% of the total nitrogen present in the 2 reactants being found in the precipitate, 0.90 ml of lysozyme solution which had been a) dissolved in 75% ethanol and heated in a boiling water bath for 2 hours,[†] b) dissolved in water and heated in the autoclave at 123°C for ½ hour,[†] c) dissolved in water and photodynamically treated (1-250,000 methylene blue and exposure of a thin layer of solution in a Petri dish to daylight for 6 days[‡]) precipitated respectively 64%, 80% and 75% of the insulin. Untreated lysozyme added in excess of that required to precipitate all the insulin (at least up to the addition of an excess of 50%) did not change the quantity of nitrogen in the precipitate, but remained in the supernatant fluid. It was also found (not recorded in the table) that the addition of twice as much insulin as was required to combine with a given quantity of lysozyme resulted in the excess remaining in solution.

Rabbits weighing 2.5 to 2.8 kg were injected subcutaneously with 0.086 ml of lysozyme-insulin suspensions containing 20 units of insulin per ml and prepared with (a) untreated lysozyme, (b) untreated lysozyme with added zinc, (c) lysozyme in 75% ethanol solution and heated as described, (d) lysozyme in water solution and autoclaved as described, (e) lysozyme photodynamically treated as described, followed by additional exposure for 18 hours to a 200 watt Mazda lamp at a dis-

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[1] Meyer, K., Thompson, R., Palmer, J. W., and Khorazo, D., *J.B.C.*, 1936, **113**, 303.

[2] Alderton, G., and Fevold, H. L., *J.B.C.*, 1945, **157**, 43; *ibid.*, 1946, **164**, 1.

* Taking the molecular weight of lysozyme as 13,900[3] and that of insulin as 32,000[4] (as found in 0.25% solution) it appears that 5 molecules of the former combine with 4 of insulin. Moisture content of the insulin was disregarded. The small quantity of zinc in the insulin (*ca.* 0.28 γ per unit) may have an influence on this ratio.

[3] Fromageot, C., and Privat de Garilhe, M., *Biochim. et Biophys. Acta*, 1949, **3**, 82; Palmer, K. J., Ballantyne, M., and Galvin, J. A., *J.A.C.S.*, 1948, **70**, 906.

[4] Gutfreund, H., *Biochem. J.*, 1948, **42**, 156, 544.

[†] Lytic power for *M. lysodeikticus* was destroyed by this treatment.

[‡] Lytic power for *M. lysodeikticus* was about 10% of the original value after this treatment.

TABLE I.

Precipitation of Insulin by Untreated and Treated Lysozyme. (1.0 ml of 0.10% solution of insulin was used in all the tests).

Ml of 0.10%-lysozyme (uncorrected for 9.54% moisture)	0.30	0.40	0.50	0.60	0.70	0.80	0.90	Treatment of lysozyme prior to its addition to the insulin
Insulin in supernate	+	+	+	+	−	−	−	Untreated (N = 17.0% on wet basis). The insulin contained 14.5% N.
Lysozyme in supernate	−	−	−	+	+	+	+	
mg N in ppt.	0.138	0.185	0.216	0.236	0.237	0.236	0.241	
% of insulin precipitated	60	80	90	?	100	100	100	
Insulin in supernate	+	+	+	+	−	−	−	Methylene blue added, used immediately after mixing.
Lysozyme in supernate	−	−	−	+	+	+	+	
mg N in ppt.	0.138	0.180	0.215	0.235	0.238	0.244	0.242	
% of insulin precipitated	60	77	90	?	100	100	100	
Insulin in supernate	+	+	+	+	+	+?	+?	Methylene blue addition + exposure to daylight for 6 days.
Lysozyme in supernate	−	−	−	−	−	−	−	
mg N in ppt.	0.112	0.145	0.174	0.206	0.220	0.242	0.261	
% of insulin precipitated	42	53	61	72	70	73?	75?	
Insulin in supernate	+	+	+	+	+	+	+	Dissolved in 75% ethanol, heated for 2 hr in boiling water bath.
Lysozyme in supernate	*	*	*	*	−	−	−	
mg N in ppt.	0.019†	0.069†	0.090	0.156	0.191	0.219	0.250	
% of insulin precipitated	?	?	?	?	50	57	64	
Insulin in supernate	+	+	+	+	+	+	+	Dissolved in water and autoclaved for ½ hr at 123°C.
Lysozyme in supernate	*	*	*	*	−	−	−	
mg N in ppt.	0.021†	0.023†	0.063†	0.167	0.206	0.256	0.269	
% of insulin precipitated	?	?	?	?	60	83	80	

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[*] Supernate, after centrifuging, is opalescent.

[†] Nitrogen in precipitate is less than in added lysozyme alone, although test for latter in supernatant fluid was negative. This is possibly owing to incomplete sedimentation of the lysozyme insulin by centrifuging; the opalescence of the supernate suggested that the complex was present in the form of particles too small to be thrown down by the centrifugal force employed.

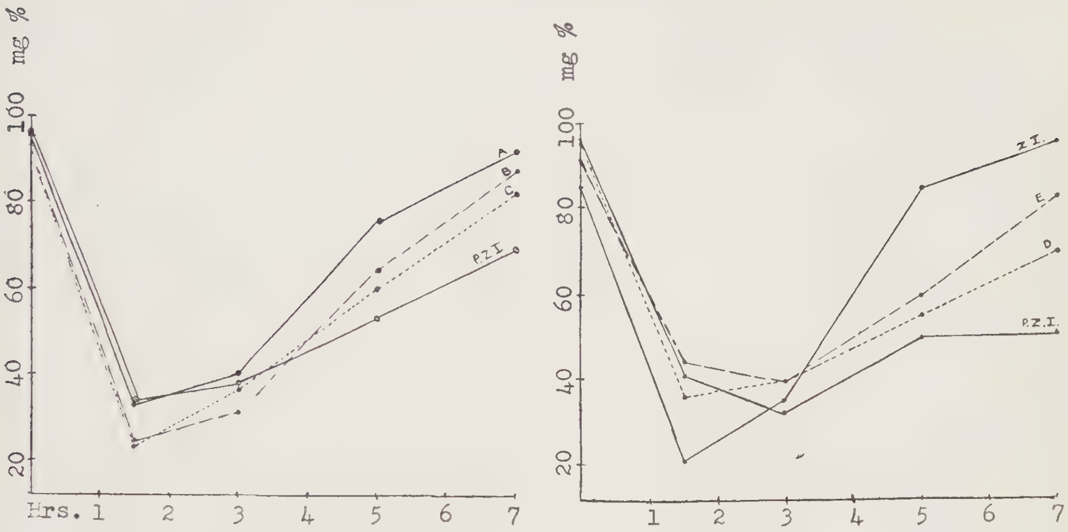

FIG. 1.

Blood-sugar curves following injection of lysozyme-zinc-insulin, protamine-zinc-insulin and zinc-insulin into rabbits. See text for description of the preparations injected.

tance of 8 inches.[§] Three γ of zinc[||] per unit of insulin were added to samples B, C, D and E.

Samples A, B and C were tested simultaneously, along with a protamine-zinc-insulin reference standard containing 40 units of U.S.P. zinc-insulin and 91.5 γ of zinc per ml,[¶] of which 0.043 ml were injected. On the second day, the remaining 2 samples of lysozyme-insulin were tested, along with the reference standard of protamine-zinc-insulin and a solution of U.S.P. zinc-insulin crystals containing 40 units per ml, of which 0.043 ml were administered. Blood was drawn from the marginal ear vein before, and 1½, 3, 5 and 7 hours after administering each of the insulin preparations to 6 rabbits, and was analyzed by the U.S.P. XIII method.

The average blood-sugar values found in the fasting state and 1½, 3, 5 and 7 hours after the injections for the 6 animals[**] in each group are shown graphically in Fig. 1. In both days' experiments the values, 1½ hours after administering lysozyme-insulin, were either approximately the same, or somewhat lower than following protamine-zinc-insulin. Between 1½ and 3 hours, 4 of the 5 curves for lysozyme-insulin and 1 for protamine-zinc-insulin were slowly rising. Five hours after the injections the blood-sugar values were, respectively, 80%, 70%, 66%, 58% and 66% of the fasting levels for samples A, B, C, D and E and 56% and 52% for the 2 protamine-zinc-insulin samples. Two hours later the corresponding figures were 98, 92, 89, 74 and 91 and 73 and 53. In the case of insulin in solution the blood-sugar was back to the fasting level in 5 hours.

If any therapeutic application is to be made of lysozyme-insulin it will have to be shown that repeated injections do not sensitize to lysozyme. Crystalline lysozyme has been reported to be mildly antigenic.[5] Less pure preparations are reported to be non-antigenic.[6] Upon gently evaporating the heated, alcoholic solution described above to dryness the lysozyme was found to be insoluble in water. This may be taken as an indication of denaturation. While a marked serological change follows denaturation it does not necessarily destroy antigenicity. More study of the antigenic properties of lysozyme seems indicated.

[§] Lytic power for *M. lysodeikticus* was about 1% of the original value after this treatment.

[||] This, and the use of 50% more lysozyme than was required to precipitate all the insulin when using untreated lysozyme, resulted in complete precipitation of insulin and the presence of lysozyme in the supernatant fluid of all the mixtures.

[¶] This is somewhat less than the 3 γ zinc per unit of insulin in samples B, C, D, and E.

[**] The results for one rabbit injected with protamine-zinc-insulin in the second group of experiments who had convulsions and died are not included in the curve.

[5] Smollens, J., and Charney, J., *J. Bact.*, 1947, **54**, 101.

[6] Kigasawa, T., *Ztschr. f. Immunitätsforsch.*, 1928, **57**, 146; Wolf, L. K., *ibid.*, 1927, **50**, 88.

The presence of lysozyme in secretions and tissues[7] suggests a possible precipitating effect on insulin *in vivo*. The solubility of the complex in serum is sufficiently great, however, to make this unlikely.

Summary. Crystalline egg lysozyme precipitates insulin. The complex produces a prompt lowering of the blood-sugar level in rabbits. Precipitates formed by mixing insulin with lysozyme which has been heated, or treated photodynamically so that 90 to 100% of its lytic activity on *M. lysodeikticus* is destroyed, but about 60% of its precipitating power for insulin is left, produce a hypoglycemic response which, up to 7 hours after injection, is intermediate between that resulting from the injection of insulin in solution and that following administration of protamine-zinc-insulin.

I wish to record my appreciation to Mr. E. K. Wolfe of the Biochemical Control Department of Sharp and Dohme, Inc., Glenolden, Pa., for performing the animal experiments.

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[7] Fleming, A., and Allison, V. D., *Brit. J. Exp. Path.*, 1922, **3**, 252.

Received October 5, 1949. P.S.E.B.M., 1949, **72**.

Renal Excretion of Digitoxin in Man Following Oral Administration.*
(17470)

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The amount of digitoxin excreted in the urine of human subjects receiving the drug has not been determined, primarily because of previous inability to measure minute amounts of any type of digitalis glycoside in biological media. However, the discovered sensitivity of the embryonic duck heart preparation[1] to minute amounts of digitoxin when present in Tyrode's solution and human serum offered the possibility that this method also could be used for the quantitative detection of digitoxin excreted in the urine of patients receiving the drug. The preliminary results of this study are reported herein.

Methods. A human urine sample of 200 ml was boiled down to approximately 50 ml on a hot plate and thereafter more slowly evaporated until the volume was reduced to

TABLE I.
Relationship Between Initial Concentration of Digitoxin in Human Urine (200 ml) and Time of Occurrence of "Digitalis Effect" in Final Extract.

Amt. dig. added (μg)	No. of extracts assayed	Avg time, "dig. effect" (min.)	S.E. of mean (min.)
1	5	—	—
2	12	47	2.28
3	10	35	1.82
4	11	27	1.45
6	9	22	1.14
8	10	17	0.73
10	5	14	0.35
12	6	11	0.20

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* Aided by grants from The Life Insurance Medical Research Fund, The United States Public Health Service, and the Sandoz Chemical Works.

5 ml. Enough diatomaceous earth (5-7 g) was added to this concentrated urine sample to form a friable powder which was dried at 75-80°C *in vacuo* (7-10 in.) for 16 hours.

The dry powder was transferred to a Waring Blender, 35 ml of $CHCl_3$ (U.S.P. grade) added, and the mixture stirred for one minute. The mixture was transferred to an Erlenmeyer flask; then the blender was rinsed with 15 ml of $CHCl_3$, which also was poured into the Erlenmeyer flask. The flask was stoppered with a cork covered with aluminum foil, and the mixture shaken for an hour, then filtered. The residue was washed 3 times with 15 ml portions of $CHCl_3$, pressing out excess solvent each time, and the combined filtrate and washings evaporated on the hot plate to a volume of 1 ml. This last quantity was quantitatively transferred to a 25 ml Erlenmeyer flask and evaporated to dryness by heating in the vacuum oven for one half hour. The dry residue was taken up in one ml of 95% ethanol by shaking for one hour, the alcoholic solution decanted to a clean Erlenmeyer flask, the original flask washed with one ml of alcohol, and the solution together with the ethanol wash dried in a vacuum oven for 30 minutes. Five ml of $CHCl_3$ were added to the dry residue, boiled down to one ml, allowed to stand for 30 minutes, decanted into a fresh Erlenmeyer flask, the original flask washed with $CHCl_3$, and the combined solvent evaporated to complete dryness in vacuum oven. Fifty ml of Tyrode's solution were added to the final residue and the flask shaken for one hour. The isolated embryonic duck heart was found to beat in this final solution as regularly and as normally as it did in pure Tyrode's solution.

Known amounts (1-12 μg) of pure digitoxin (Sandoz) then were added to 200 ml quantities of human urine and the urines were extracted as above. It was found that the final extract produced a "digitalis effect"[2] if two or more micrograms of digitoxin were added to the original 200 ml of urine. Moreover, the

TABLE II.
Urinary Excretion of Digitoxin in Normal Human Subjects.

		First 24 hr			Second 24 hr			Third 24 hr		
	Age	U.V. (ml)	Time of "dig. effect" (min.)†	Tot. amt. dig. excr. (μg)	U.V. (ml)	Time of "dig. effect" (min.)†	Tot. amt. dig. excr. (μg)	U.V. (ml)	Time of "dig. effect" (min.)†	Tot. amt. dig. excr. (μg)
	39	1450	21(6)	44	1500	19(8)	60	1700	13(10)	85
	31	1720	14(10)	86	2250	24(6)	68	940	19(8)	38
	35	2000	20(6)	60	1210	14(10)	60	970	26(4)	19
	30	2470	10(12)	144	1050	23(6)	30	520	46(2)	5
	24*	1700	12(12)	102	1930	20(6)	58	760	23(6)	23
Avg	32	1868	15	87	1588	20	55	978	26	34

* Female.

† Figures in parentheses represent calculated amount of digitoxin (μg) in original 200 ml quantity of urine.

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[1] Bine, R., Jr., and Friedman, M., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 487.

[2] Friedman, M., and Bine, R., Jr., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 533.

time of occurrence of the "digitalis effect" in the duck hearts was dependent upon the amount of digitoxin added. Thus, standards (Table I) were obtained. Such standards were found to be necessary because of the significant but relatively fixed loss of digitoxin in the extraction of such large quantities of urine.

For the assay of urinary excretion of digitoxin, control urine samples (200 ml) were obtained from 4 young males and one female subject (average age 32 years). These were extracted and tested on the duck hearts as described above and found to produce no effect. Each subject then was given 1.2 mg of digitoxin by mouth within a period of 6 hours. Urine collections subsequently were obtained. The daily volumes were measured, and then 2 separate 200 ml samples of each were extracted and assayed on 6-8 duck hearts. The average time of occurrence of the "digitalis effect" of the 2 samples was compared to the standard values. In this manner the amount of digitoxin originally present in a 200 ml quantity of urine could be approximated. The total daily excretion then was calculated by multiplying the amount of digitoxin in the 200 ml quantity of urine, by the total daily urine volume/200.

Results. Each of the 5 subjects (Table II) excreted greatly varying amounts of digitoxin in his urine after the oral ingestion of 1.2 mg of the drug. During the first 24 hours after ingestion an average of approximately 87 μg (Range: 44-144) or 7% of the administered dose could be detected in the urine. The second day's urine of each subject contained somewhat less digitoxin (average: 55 μg). The third day's urine of 4 subjects tested contained appreciably less digitoxin (average: 21 μg). These results indicate that approximately 14% of the amount of digitoxin administered to these 5 subjects could be recovered in the urine collected the first 3 days after its administration.

Summary. The quantitative detection of orally administered digitoxin in the urine of subjects receiving the drug has been accomplished. Approximately 14% of the given dose is excreted in the urine of normal subjects during the first 72 hours after its oral ingestion.

The authors wish to express their indebtedness to Maude Gardner and Nancy Bryant for their invaluable assistance.

Received October 20, 1949. P.S.E.B.M., 1949, **72**.

Adrenotrophic Activity of Human Urine.* (17471)

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The ability to determine adrenocorticotrophic hormone in body fluids of man would be a valuable aid in extending our knowledge of pituitary-adrenal physiology. Although there have been previous reports dealing with the presence of adrenotrophin in urine, the results (summarized in Table I) are conflicting. Part of this confusion arises from the failure of some of the investigators to use hypophysectomized test animals, an omission which unfortunately invalidates their conclusions. The remaining confusion apparently cannot be so explained. For example, Jones and Bucher,[3] using a specific assay method, found no adrenotrophic activity in 200 to 440 ml of urine of normal persons, while Reiss and associates[5] and Williamson[6] have reported the

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* Abridgment of thesis submitted by Dr. Locke to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Master of Science in Medicine.

† Deceased.

[1] deBoissezon, P., *Bull. d'histol. appliq. a la physiol.*, 1936, **13**, 129.

TABLE I.
Tests for Adrenotrophic Activity in Human Urine.

Reference	Source	Preparation	Total vol. per animal, ml	Assay method	Adrenotrophic activity
DeBoissezon[1] (1936)	Pregnancy	Untreated	16	Incr. in adrenal wt and cholesterol in intact guinea pig	Yes
Paschkis, Rakoff, and Cantarow[2] (1942)	Normal	70-86% ethyl alcohol precipitate	Not stated	Incr. in adrenal wt in intact 2-day-old chicks and 4-day-old rats	Yes
Jones and Bucher[3] (1943)	Normal	Fractional ammonium sulfate precipitates	200–440	Increase in adrenal wt of hypophysectomized rat	No
Blumenthal[4] (1945)	Pregnancy, normal female	Untreated, fresh and preserved	4–16	Incr. mitosis in adrenals of intact guinea pig	Yes
Reiss, Peglar, and Golla[5] (1946)	Pregnancy	1. Untreated 2. Benzoic acid adsorbed 3. Picric acid ppt.	Not stated Not stated 25	Disappearance of sudanophobe zone in adrenal of hypophysectomized rat	1. No 2. No 3. Yes
Williamson[6] (1946)	Normal female	1. Untreated 2. Dialyzed 3. Dialyzed, acetone precipitated 4. Dialyzed pervaporated and acetone precipitated	4 4 120–460 30	Incr. in adrenal wt in intact rats Ascorbic acid depletion of adrenals of hypophysectomized rat	1. Yes 2. Yes 3. Yes 4. Yes
Cooke, Graetzer, and Reiss[7] (1948)	Normal	Permutit adsorbed	250	Ascorbic acid depletion of adrenals of hypophysectomized rat	Yes

presence of adrenotrophic activity in about 25 ml of urine of normal and pregnant humans. Since our work was completed, Cooke and associates[7] have reported positive reactions for adrenotrophin in concentrates representing 250 ml of normal urine, prepared by an adsorption technic.

Because of these conflicting results and because of some claims that the presence of adrenotrophin in body fluids in Cushing's syndrome is of etiologic significance, we have reinvestigated this problem using several different methods of concentrating the urine, and have placed particular emphasis on the study of urine from such pathologic states as may conceivably be associated with extremes of adrenotrophic activity. Although in general, our results have been negative, we are reporting them briefly, since they may be of some interest and of some use to others interested in the clinical-laboratory aspects of pituitary-adrenal disorders.

Material and methods. Adrenotrophic activity was determined by the ascorbic acid depletion method described by Sayers, Sayers and Woodbury[8] modified by: (1) the use of hypophysectomized male rats of the Wistar strain rather than the Sprague-Dawley, and (2) the injection of test material into the jugular vein or peritoneum, rather than the tail vein. In most instances 5 rats were used for each sample of urine. Ascorbic acid concentration of the rat adrenal glands was determined according to the procedure described by Roe and Kuether,[9] adapted for use with tissue. The solutions were read in a Coleman, Jr. spectrophotometer at 510 λ and the adrenotrophic activity was expressed in terms of the reduction of ascorbic acid concentration (milligrams per 100 g fresh tissue) in the right gland, removed one hour after injection, as compared to the left gland, removed prior to the administration of the urine preparation. This calculation is subsequently designated as Left—Right, or L—R.

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[2] Paschkis, K. E., Rakoff, A. E., and Cantarow, A., *Endocrinology*, 1942, **30**, 523.

[3] Jones, G. E. S., and Bucher, N. L. R., *Endocrinology*, 1943, **32**, 46.

[4] Blumenthal, H. T., *J. Lab. and Clin. Med.*, 1945, **30**, 428.

[5] Reiss, Max, Peglar, Harold, and Golla, Y. M. L., *Nature*, 1946, **157**, 413.

[6] Williamson, M. B., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 191.

[7] Cooke, D. S., Graetzer, E., and Reiss, M., *J. Endocrinol.*, 1948, **5**, lxxxix.

Urine was obtained from a variety of normal and abnormal subjects and prepared by one of the following methods: (1) fresh unconcentrated urine, or fresh dialyzed urine; (2) urine dialyzed and pervaporated to one-fifth volume, after which it was precipitated by means of acetone (Williamson,[6]); (3) ultrafiltration of urine through a collodion membrane at 60 to 80 lb pressure, after which the membrane was dissolved and the residue was extracted with 0.1 N sodium hydroxide and neutralized (Gorbman[10]); and (4) urine precipitated by 4 volumes of acetone at pH 3.5 and the precipitate extracted with 0.1 N sodium hydroxide, neutralized and dialyzed (Frank and associates[11]).

In order to obtain control values for comparison with experimental values, solutions containing no adrenotrophic activity (0.9% saline solution; distilled water) were administered to 25 hypophysectomized rats in volumes of 1 to 10 ml according to the procedure described previously. The mean concentration of ascorbic acid in the right gland was found to be higher than the left by 4 ± 3.2 mg, or a mean L—R of –4 ± 3.2. These results are much more variable than those reported by Sayers and associates[8] which indicate no difference in ascorbic acid concentration between left and right glands.

Ordinarily the urine or urinary concentrate was administered to 5 rats, but owing to toxicity not all the animals survived each experiment. Only those results in which 2 or more animals survived so that a mean value could be determined are included. Because of the variability of the animals, already mentioned, no mean L—R value less than 25 mg was considered significantly different from the controls, this value representing the minimal level for a test of significance between means. That toxicity of the concentrate did not impair the action of adrenotrophin was assured by dissolving pure adrenotrophin in the concentrate and observing the response as compared with the same amount of hormone in saline. Under these conditions, no difference in the L—R values was observed.

Results. Fresh, unconcentrated or dialyzed urine. Williamson[6] reported that 4 ml of fresh urine possessed adrenotrophic activity as measured by the increase in adrenal weight in the intact rat. Urines obtained from 3 normal persons, one patient with Cushing's syndrome and one patient with somatic and sexual precocity were inactive when tested in hypophysectomized rats in amounts of from 2 to 10 ml of urine per rat. Thus, the results obtained with intact rats would appear to be nonspecific responses.

Dialysis and pervaporation method of Williamson. Fresh mixed female urine prepared by dialysis, pervaporation and acetone precipitation was reported by Williamson to induce an L—R value of 145 mg when administered in an amount of 30 ml equivalent of urine. This method was repeated using from 14 to 88 ml equivalent of urine with entirely negative results.

Ultrafiltration method. The method described by Gorbman[10] for the recovery of urinary gonadotrophin was tested to determine whether adrenotrophin, if present, could also

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[8] Sayers, M. A., Sayers, George, and Woodbury, L. A., *Endocrinology*, 1948, **42**, 379.

[9] Roe, J. H., and Kuether, C. A., *J. Biol. Chem.*, 1943, **147**, 399.

[10] Gorbman, Aubrey, *Endocrinology*, 1945, **37**, 177.

[11] Frank, R. T., Salmon, U. J., and Friedman, Reuben, PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1666.

TABLE II.
Assays for Adrenotrophic Activity in the Urine of Various Endocrinopathies.

No. of patients	Diagnosis	No. of assays	Adrenotrophic activity Positive (L—R >25 mg)	Doubtful (L—R = 20-25 mg)	Negative (L—R <20 mg)
4	Cushing's syndrome, Adrenal cortical hyperplasia	9	1	3	5
2	Hypopituitarism	2	0	1	1
1	Addison's disease	1	0	0	1
2	Sexual and somatic precocity	2	1	0	1
1	Natural menopause	1	1	0	0
3	Surgical menopause	3	1	0	2
1	Ovarian dwarfism	1	0	0	1
1	Pregnancy 5 mo.	1	0	1	0
1	Pituitary tumor	1	1	0	0

be recovered. Concentrates equivalent to 15% of the total 24-hour specimen, obtained from menopausal and pregnant women, states in which urinary adrenotrophin had been previously detected, gave negative results.

Acetone precipitates of urine. The precipitation of urine by means of acetone and the extraction of the precipitate yield fairly good recoveries of urinary gonadotrophin (Frank and associates[11]). This method was investigated extensively for adrenotrophic activity. Twenty-four-hour urine specimens were collected from 7 normal subjects (4 females and 3 males) and 10 tests were carried out. Concentrates ranging from 2.5 to 23% of the total 24-hour specimen were injected per rat. Concentrates equivalent to 2.5 to 10% of the total specimen gave negative results. However, the equivalent of from 10 to 23% of the specimen per rat yielded positive L—R values (more than 25 mg) in 2 instances, borderline responses (L—R of 20 to 25 mg) in 2 instances, and negative responses in 2 instances. Thus, while far from consistent, these results indicate at least that an amount equivalent to 10% or more of the 24-hour specimen was necessary to induce positive responses.

Since adrenotrophic activity could not be demonstrated consistently in normal urine, tests were performed on urines which might conceivably be richer in adrenotrophin, that is, in certain endocrine disorders. Among the endocrinopathies which might be associated with extremes of adrenotrophic activity, we tested the urines of patients with Cushing's syndrome, hypopituitarism, Addison's disease, in the menopause, and with a few miscellaneous disorders (pituitary tumor, sexual and somatic precocity and ovarian dwarfism). All tests were performed with amounts of urine representing 10% or more of the entire 24-hour specimen. The results of these assays are summarized in Table II.

Comment. The attempts to demonstrate adrenotrophic activity in human urine of both normal and pathologic states have been generally unsuccessful. We have been unable to confirm the presence of adrenotrophin with consistency, although occasional specimens have indeed given positive results. While it may be possible that adrenotrophin is either not excreted in the urine or excreted in biologically inactive form, the few occasional positive results encountered by us as well as by other investigators would seem to overrule this possibility. Another possibility is that adrenotrophin is excreted in biologically active form but as a degraded part of the protein molecule. Such a possibility might explain why most of the methods used, designed to concentrate the urinary protein, do not yield adrenotrophic activity with consistency.

Assuming that adrenotrophin is excreted in human urine, the problem of its detection might be resolved in two ways. The first approach would be to increase greatly the sensitivity of the bioassay procedure so as to permit the use of fresh urine. Were it possible to obtain positive results with a few milliliters of fresh urine, it would then be relatively simple to work out systematically a technic for the concentration and purification of the hormone from urine. However, since the present Sayers' assay procedure is already extremely sensitive (in our hands a minimum of 0.8 μg of pure adrenotrophin was capable of inducing significant L—R values) it seems quite unlikely that any new assay method sufficiently sensitive to permit the use of fresh urine will be soon available.

The alternative approach would be to continue the empirical attempts to purify and concentrate the urine in the hope that a method will be found to permit the administration of large volumes of urine without toxicity to the test animals or appreciable loss of adrenotrophic activity. As such, the method described by Cooke and associates may represent a solution.

Summary. Using a specific and sensitive assay procedure, we have tested the urine of normal persons and of patients having a variety of disease states for its adrenotrophic activity. Tests using both fresh urine and urine concentrates prepared in three different ways were carried out. Contrary to previous reports, no adrenotrophic activity could be consistently demonstrated.

Received Sept. 13, 1949. P.S.E.B.M., 1949, **72**.

Plasma Levels of Radioactive Iodine (I^{131}) in Human Tracer Studies. (17472)

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The purpose of the studies reported here was to examine the post ingestion plasma radioiodine curves in euthyroid, hypothyroid, and hyperthyroid subjects as a first step toward analysis of the various factors regulating the levels of such plasma iodine curves.

Methods. Patients were selected after previous clinical and laboratory study had established the presence of a euthyroid, hypothyroid or hypothyroid state. All were in an untreated state. They were given 100 microcuries of radioiodine by mouth before breakfast. The dosage was calculated on the basis of the shipping tag from Oakridge Laboratories. No carrier iodine was added. Each dose was given with 50 cc of distilled water and followed by two 50 cc portions of water to wash the container and mouths of the subjects. Blood specimens were drawn at 30 minutes, 1, 3, 6, 12, 24, and 48 hours; the blood was oxalated and centrifuged at 2500 rpm for 10 minutes. Then 0.2 cc of plasma was pipetted into shallow cups containing one drop of phenolphthalein, 0.2 cc of 0.75 N NaOH and 0.2 cc of detergent (Orvus). Duplicate samples were dried under an infra-red lamp and counted with a bell type Geiger Müller tube (Cyclotron Specialties Co., Model 410-A) and counter (Technical Associates Model GS4). Duplicate specimens of reagents alone, exclusive of plasma, were checked for radio-contamination The counts were corrected for decay with reference to the day of ingestion. Eleven cases of untreated myxedema and 14 non-myxedematous cases (5 hyperthyroid and 9 euthyroid) were so studied.

Results. When the data for the entire 25

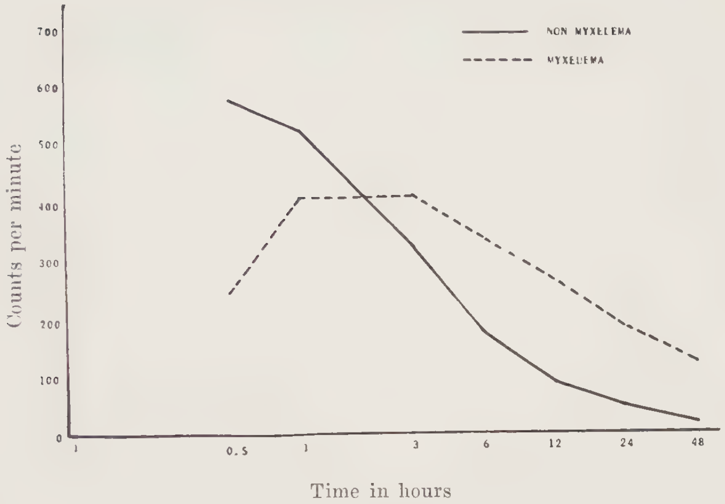
Plasma Levels of I^{131}

FIG. 1.

Combined plasma curves of the geometric means of the entire myxedematous and non-myxedematous groups.

patients were analyzed, two curves could be obtained, one for the myxedematous and one for the non-myxedematous patients.* Fig. 1 shows these curves; here the geometrical mean (calculated by probit analysis) of counts per minute is plotted on an arithmetical scale against time on a logarithmic scale.

The data from which these curves were obtained are shown in Table I.

Discussion. The major differences noted between these 2 groups of patients (myxedematous and non-myxedematous) may be listed as follows: 1. An early, high peak in the plasma radioactivity of the non-myxedematous group. 2. A later, lower peak in the plasma radioactivity of the myxedematous group. 3. A slower decrease in radioactivity during the first 48 hours in the myxedematous group. These differences appear valid; it seems unlikely that the effect of plasma volume or pre-existing iodine content of plasma would be the cause of these changes. It is more reasonable to suppose that the variations in these two curves are caused by: (a) different rates of absorption of radioiodine from the gastro-intestinal tract, (b) varying rates of excretion of radioiodine by the kidney, (c) speed of formation and storage of thyroid

TABLE I.

Plasma Radioactivity, as Counts Per Minute of the Three Groups of Patients Studied. The individual patient's curve is read horizontally under the time intervals shown.

30″	1′	3′	6′	12′	24′	48′
			Euthyroid			
398	350	218	120	62	10	0
460	372	231	129	62	19	0
518	398	291	170	71	25	0
600	446	313	172	90	18	4
635	480	363	194	91	31	7
671	530	363	231	118	45	20
704	638	443	247	128	70	24
731	676	499	248	128	121	63
860	822	636	272			
			Hyperthyroid			
280	400	148	63	18	16	42
551	417	258	125	57	57	48
555	496	321	190	136	137	195
813	911	385	225	148	183	218
		580	289	198	184	
			Myxedema			
75	212	296	252	184	91	19
88	223	319	253	197	116	31
158	272	349	291	199	117	50
275	345	373	304	202	139	121
312	384	381	393	213	163	125
315	459	396	336	220	216	167
336	465	416	346	274	247	190
347	522	432	358	307	278	199
397	541	493	368	320	325	205
400	568	541	400	385	364	278
542	592	543	565	475		

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* The authors are indebted to Frederick J. Moore, M.D., for the statistical analysis of the data.

hormone, (d) re-entry of radioiodine into the blood stream in a protein bound form. How much of a role each one of the above factors might play in the ultimate derivation of the plasma curve requires further study.

While this work was in progress McConahey *et al.*[1] published the results of a similar study of serum radioactivity in hyper-, hypo-, and euthyroid individuals, after ingestion of I^{131}. They reported experiments on 31 patients divided as follows: 17 with hyperthyroidism, 9 euthyroid, and 6 myxedematous (one patient was studied twice, once while euthyroid and then after a "thyroidectomizing" dose of I^{131}). Their patients did not receive similar doses of radioiodine but rather there was a range from 0.1 to 100 millicuries of the drug. McConahey *et al.*[1] felt that there was little if any disturbing radiation effect on the plasma curves from the large doses given to some patients.

The principal finding of difference in McConahey's work from the work presented here, is the failure of our figures to differentiate hyperthyroid and euthyroid individuals. It seems reasonable to suppose that hyperthyroid individuals would show a different rate of increment and decrement of radioiodine in blood after an oral dose than would euthyroid individuals. The differences obtained here, however, were not sufficient to be used for diagnostic purposes and probit analysis of the data would not significantly separate the two groups in the time periods studied.

Summary. Curves of plasma radioactivity were determined for 25 patients after the ingestion of 100 microcuries of radioiodine. The patients were divided into 11 cases of myxedema, 5 cases of hyperthyroidism and 9 euthyroid individuals. The curves were constructed from plasma samples drawn at 30 minutes 1, 3, 6, 12, 24, and 48 hours. It was not possible to statistically separate, at these time intervals, the curves of the hyperthyroid and euthyroid individuals. The curves of the myxedematous patients, however, were significantly different from those of the non-myxedematous patients; thus the myxedematous curves show a later peak level, a lower peak level and a fall more delayed than do the curves of the non-myxedematous patients.

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Received August 18, 1949. P.S.E.B.M., 1949, **72**.

Acute Adaptation of Rats to Very Low Oxygen Pressure.* (17473)

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In a recent publication[1] the author reported evidence of depressed activity of the thyroid gland in anoxic rats. This thyroid depression may act as an adaptive change which would aid the accommodation to anoxia. Himwich[2] called attention to the evidence of Barach and others[3,4] which indicated that thyroidectomy increases the tolerance of rats to anoxia. Therefore, if anoxia were to result in a degree of functional thyroidectomy, this latter condition should increase the tolerance to more severe anoxia.

The experiments described here indicate that if normal rats are first exposed to anoxia for 2 or more hours at a low temperature, they can then withstand severe anoxia as readily as has been reported for surgically thyroidectomized rats.[3,4]

Method. The low pressure chambers were constructed from 9 liter vacuum desiccators.

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* This investigation was aided by grants-in-aid from the National Research Council Committee on Endocrinology and the Atomic Energy Commission.

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[2] Himwich, H. E., personal communication, 1949.

[3] Barach, A. L., Eckman, M., Molomut, N., *Am. J. Med. Sci.*, 1941, **202**, 336.

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TABLE I.
Adaptation and Its Effect on Hours Survival at Low Air Pressure.

No. rats	Time at 268 mm Hg, 14°C,* hr	Time at 162 mm Hg, 14°C,* hr	Time at 162 mm Hg, 29-31°C, hr	Remarks
6 ♂	4	5		All lived
2 ♀	2	8		One died at end
3 ♂	4	2	3	All lived
7 ♀	2	1.5	4.5	Two died
		Controls		
5 ♂ and ♀		.12 ± .06		All died
1 ♀		1.0		Removed alive
17 ♂			.20 ± .18	All died
24 ♀			.25 ± .20	” ”

* Temperature range 11° to 15°C.

They were continuously ventilated at the rate of 2 liters of air per rat per minute. The flow rate was measured at 760 mm Hg. The chambers could be operated either within a refrigerator or at room temperature. The experimental rats were Long-Evans strain, unfasted, male and female, old and young adults.

Results. Control rats were placed within the chambers at various temperatures (14-31°C) and the air pressure reduced within 3 minutes to 162 mm Hg. Within 6 to 15 minutes these rats developed convulsions and died. The "adaptation" experiments were conducted with the chambers placed within a refrigerator at 14°C (range 11° to 15°C) and the pressure reduced within 90 seconds to 268 mm Hg and maintained at that pressure for 2-4 hours. Then the pressure was further reduced to 162 mm Hg (14°C) and these conditions were maintained for 1.5-2 hours. At this point some of the chambers were removed to room temperature (29-31°C) and others were left within the refrigerator, the air pressure in both sets of chambers was still maintained at 162 mm Hg (oxygen pressure 32 mm Hg). After 4-6 additional hours under these conditions, 17 out of 20 rats remained alive and they were returned to sea level for further observation. These rats therefore endured for 5-8 hours anoxic conditions which killed 98% of controls in 10-15 minutes and this high degree of tolerance was developed in a 3-4 hour adaptation period.

The permanency of this adaptation was tested by the following experiment. Two rats were exposed to cold and anoxia as outlined in the above paragraph. The air atmosphere at 30°C was then replaced by 150 mm of pure oxygen, which is approximately equivalent to the sea level pressure of oxygen. After 4 hours the oxygen atmosphere was replaced by air at 162 mm and the animals succumbed in 12 and 15 minutes, essentially the same as unadjusted controls. Therefore, only 4 hours of oxygenation was adequate to destroy the tolerance which these rats had developed.

Of 20 rats adapted to anoxia as outlined above, only 3 died when exposed continuously to air at 162 mm for 5-8 *hours* (Table I). Of 46 *control* rats at the same pressure, 45 died in 4-27 *minutes* (0.06-0.45 hour) and one lived for one hour (Table I). Low environmental temperature was important in developing the tolerance of the adapted group, but the control rats died at a variety of temperatures.

The adapted animals were allowed to recover at sea level and room temperature. Their rectal temperature was 19-20°C if the entire exposure to cold and anoxia had been at 14°C. If the last 4-6 hours of anoxia was at room temperature, the rectal temperature was 1-2°C above room temperature (29-31°C). These animals were quite refractory to mechanical stimuli for the first 5-15 minutes following the anoxia period. However, within 2-3 hours, they would often eat and drink and within 24 hours they appeared normal. All of these animals were still alive one month after the experiment.

The eyes of the adapted animals showed striking changes. After 3 or more hours at an air pressure of 168 mm Hg the eyes were clouded by severe cataracts. Examinations of the lens directly by excision and indirectly by the slit lamp† showed a well developed subcapsular cataract. Seventy percent (14 cases) of the severely anoxic rats developed cataracts during the period of anoxia but all of the cataractus lenses became clear within 60-120 minutes when the animals were allowed to recover at sea level.

Discussion. The acute adaptation to anoxia described here differs from the usual chronic acclimatization to high altitude. In the latter the subjects are said to live fairly normal lives (reviewed by Van Liere[5]) and the acclimatizing process requires weeks to develop[6] although it probably requires generations to become complete.[7] Furthermore, on return to sea level condition, the loss of true acclimatization is slow.[8] In our experiments the animals exhibited a very low level of existence but their tolerance to extreme anoxia developed within a few hours. On return to sea level oxygen pressure the adaptation was lost within a few hours or about as rapidly as it had developed.

This acute adjustment to severe anoxia may involve some or all of the following factors: (a) reduction of body temperature, (b) reduction of metabolic rate, (c) reduction of thyroid activity, (d) anaerobic metabolism. Evaluation of these possible factors must await additional experimental investigation.

It is possible that the adjustment to anoxia as illustrated in this report could have no significant relation to the thyroid gland, but in view of recent work[1] anoxic suppression of the thyroid appears to offer a plausible explanation for the acute adjustment to severe anoxia.

Britton and Kline[9] investigated the effect of temperature on survival of asphyxiated adult rats. Their results demonstrated that animals being asphyxiated in sealed chambers survived 2-3 times longer at 16-18°C than at a temperature of 31-32°C.

Previous investigators[10-12] have increased the tolerance of rats to anoxia by the administration of antithyroid drugs for 8 or more days preceding the low pressure exposure. In the experiments of Barach *et al.*[3] thyroidectomized rats were found to withstand 160 mm Hg for 2 hours (temperature not reported). However, Leblond[12] showed that such thyroidectomized rats must be well fed in order to withstand severe degrees of anoxia. The adapted animals described in the present work easily tolerated the severe anoxic conditions reported for hypothyroid or thyroidectomized rats.

Previous investigators[13,14] have observed cataracts from anoxia. The animals of Bellows and Nelson[13] were exposed to 225 mm until 50 percent of the rats were dead, after which 10% of those surviving had cataracts. Comparison of previous results with these reported here shows that in our experiments the anoxia was more severe, a greater number of rats survived the experiment, and more animals developed cataracts.

Conclusion. Rapid adaptation occurs in adult rats exposed to anoxia at low temperatures. This enables the animals to tolerate extreme anoxia (32 mm Hg pressure of O_2) at various temperatures (14-31°C) for a period more than 40 times longer than unadapted controls.

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† These slit lamp observations were made by Dr. J. W. McKinney, Department of Ophthalmology, University of Tennessee College of Medicine.

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Received October 24, 1949. P.S.E.B.M., 1949, **72**.

Function of Egg White in Embryonic Development. (17474)

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In an attempt to decide whether or not the yolk or the egg white is the carrier of a growth factor transmitted through the egg from the hen's feed to the chick, the experimental technic described here was devised. In the past it has been assumed that egg white has a purely nutritional function in the growth and development of the chick embryo, but no experimental evidence exists to support this supposition.

The yolk also furnishes food, but in addition the yolk-sac serves as an organ for the digestion and assimilation of food by the chick embryo. This function of the yolk-sac indicates a structural relationship between the embryo and the yolk.

In this experiment fertile hen's eggs from crosses of Rhode Island Reds and White Leghorns were used. A circular window approximately ¼ inch in diameter was bored in the small end of each egg and the egg-white was removed into a glass ground-joint wash bottle by suction. To combat bacterial infection 0.1 cc of an aqueous solution containing 3000 I.U. of penicillin and 3.3 mg of streptomycin was injected into each of the eggs so treated. The egg white from another fertile egg was then transferred by air pressure from the wash bottle into the egg from which the egg white had previously been removed. When one egg white was not sufficient to completely refill the shell, that from another fertile egg was added. Thus, at times egg white from more than 1 egg was used for replacement in a single egg.

After the egg was completely filled with egg white, the window in the shell was closed with a strip of scotch tape and the egg placed into an incubator for 3 weeks. At intervals of 7 days, the condition and development of the chick embryo was observed by candling the eggs.

In the first successful series, 12 such eggs were prepared; of these 3 chicks hatched which were normal in every respect. Of the rest of the embryos, two lived 2 days, one 4, two 6, one 7, one 13, one 15 and one 21 days.

These are probably the first live chicks produced after such drastic treatment. This experiment demonstrates that the chief function of the white of the hen egg is mainly to furnish food for the developing embryo.

It has been previously held that the two chalazae serve to orient the embryo in a proper position for hatching by keeping the yolk more or less fixed. In these experiments, however, the two chalazae were removed with the egg white and thus the position of the developing embryo depended upon gravity. Further work is in progress on the nutrition of the chick embryo employing this technic.

Received October 20, 1949. P.S.E.B.M., 1949, **72**.

Distribution of Vitamin B_{12} in Natural Materials.* (17475)

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The increasing use of vitamin B_{12} in the treatment of human macrocytic anemias and in certain natural rations for farm animals makes the knowledge of the distribution of this vitamin in biological materials of real importance. A rat assay for vitamin B_{12}, based on the fact that thyroid active materials increase the requirements for this factor, has been used with considerable success in our laboratory.[1,2] With the availability of pure vita-

TABLE I.
Vitamin B_{12} Content of Natural Materials.

Daily supplement	Avg growth 2 week assay period, g	Min. vit. B_{12} content fresh basis, μg/100 g	Min. vit. B_{12} content dry basis, μg/100g
None	35 ± 5		
0.1 μg vit. B_{12}, inj.	66		
0.1 μg vit. B_{12}, orally	65		
1 mg thymidine, inj.	36		*
2 mg thymidine, orally	36		*
250 μg thymine, uracil, adenine, guanine, and 500 μg rutin and hesperidin	35		*
10 ml tomato juice, fresh	34	*	
6% dried streptomycin "slop"	95		>22
6% condensed fish solubles	96	20	>40
5% herring stickwater	55	7	14
5% desiccated hog mucosa	43		trace
2% desiccated swine adrenals	57		15
1.25% desiccated sheep rumen contents	76		50
2.5% desiccated sheep rumen contents	93		50
10% crude casein	53		3
10% purified casein (vitamin test)	15		*
10 ml raw milk (cow)	41	trace	
20 ml raw skimmed milk (cow)	42	trace	
25 ml raw milk (goat)	28	*	
15% whole milk powder (Klim)	61		2.5
Rennet ppt. casein 25 g milk†	50	0.12	1
15% malted milk	54		2.2
2.5 g cheese cheddar	56	1.4	2.3
4 g egg yolk	64	1.4	2.8
1 g beef liver (fresh)	82	15	>50
1 g beef kidney (cooked)	90	20	>50
.125 g viobin liver powder, 37°‡	80		100
.125 g viobin liver powder, 70°C‡	78		97
5 g beef round, sample 1 (cooked)	74	2	3.6
5 g beef round, sample 2 (cooked)	83	3	5.5
2.5 g beef round, sample 2 (cooked)	68	3	5.5
5 g beef tongue (cooked)	91	3	5.5
.75 g chicken liver (fresh)	70	11	35
5 g pork shoulder, sample 1	73	2	3.6
5 g pork shoulder, sample 2	67	1.2	2.2
5 g pork hocks	51	0.5	0.9
5 g pork ham	68	1.2	2.2
5 g mutton	82	3	5.5
5 g veal	76	2	3.6
5 g horsemeat (canned)	84	3.4	7.5

* No measurable quantity.
† Calculated on fresh milk basis.
‡ Defatted and desiccated.

min B_{12} it is possible to supply standard amounts to rats and thereby calculate the actual amount of vitamin B_{12} present in the samples tested.

We wish to present in this paper a preliminary report of the distribution of vitamin B_{12} in a variety of biological materials.

Experimental and Results. The assay procedure has been described in detail in a previous paper.[1] Male weanling rats are placed on a corn-soybean-basal ration containing 0.1% iodinated casein† for a 2 week depletion period. At the end of this time supplements are administered for another 2-week period during which time the growth response is followed. The vitamin B_{12} content of the sample is calculated through the use of a standard growth curve plotted from data obtained with graded levels of crystalline vitamin B_{12}.[2]

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* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by grants from the National Livestock and Meat Board and from the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

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[2] Register, U. D., Lewis, U. J., Thompson, H. T., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 167.

† Supplied by Dr. W. R. Graham, Cerophyl Laboratories, Inc., Kansas City, Mo.

Five rats were used for each assay and an average of the growth response for these rats was taken. In most cases the material to be tested was added on a percentage basis to the basal ration and the vitamin B_{12} content was calculated from the total food consumed. In other cases, due to the nature of the material, the supplement was given in separate food containers and the amount consumed was measured directly. The results are summarized in Table I.

Average growth responses on the basal ration during the 2 week assay period have been found for a large number of animals to be 35 g (± 5 g). The daily administration of 0.1 μg of vitamin B_{12} either orally or by injection, increased the growth response to 65-66 g. Since Hall, *et al.*[3] showed that incubation of vitamin B_{12} with gastric juice increased the hematopoietic activity in pernicious anemia when administered orally, a similar treatment was tried for the rat. No effect on the activity of vitamin B_{12} was noted after incubation with human gastric juice.

Thymidine has been found to be capable of replacing vitamin B_{12} in the nutrition of *Lactobacillus lactis*.[4,5] Results presented in Table I show that this compound has no activity in the rat assay. Fresh tomato juice, which has been found to contain the TJ factor required by *Lactobacillus lactis* Dorner,[6] was likewise found to be inactive. Furthermore, a combination of thymine, uracil, adenine, guanine, rutin and hesperidin produced no growth response. It appears, therefore, that the rat assay is rather specific for vitamin B_{12}.

Two samples of yeast autolysates, U2 and Z7, supplied by Dr. C. N. Frey, Standard Brands, Inc., were found to be completely inactive. A sample of "slops" from streptomycin production was found to be very high in vitamin B_{12}. The regular commercial condensed fish solubles showed high activity while a sample of herring stickwater was not as active.

Sheep rumen contents were very active, which suggests a synthesis of this vitamin within the rumen. Milk, cheese, meats and egg yolk were found to be relatively good sources of the vitamin. Dried soybean sprouts, barley, fresh potatoes, beans, cabbage, green peas, alfalfa leaf meal, and fresh grass contained no significant amount.

The vitamin B_{12} content of beef kidney was found to be as high as that of beef liver. This is in agreement with previous results obtained on the vitamin B_{12} content of liver and kidneys from rats fed different rations.[7] The higher values obtained with the two liver powder preparations of the VioBin Corporation may be attributed to the fact that they were defatted and also that the fresh liver was fed at a near maximum level. However, more samples must be tested before definite conclusions can be made. It can be seen from the results that drying the liver at 70° did not noticeably alter the vitamin B_{12} content. The variations in pork samples obtained in this work and in previous work[2] are undoubtedly due to the fact that swine are monogastric animals and therefore do not benefit from the nutrients that may be produced by microorganisms in the rumen of cattle. The high level found in horse meat indicates that the synthesis of vitamin B_{12} in the cecum of the horse may be as efficient as in the rumen of cows and sheep.

Summary. The vitamin B_{12} content of a number of natural materials as determined by the rat assay is presented. Fish solubles, streptomycin "slops", sheep rumen contents and glandular meats are excellent sources of vitamin B_{12}. Muscle tissue, eggs and milk products contain lesser amounts, whereas plant materials show no measurable activity.

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[3] Hall, B. E., Morgan, E. H., and Campbell, D. C., *Proc. Staff Meet., Mayo Clin.*, 1949, **24**, 99.

[4] Shive, W., Ravel, J. M., and Eakin, R. E., *J. Am. Chem. Soc.*, 1948, **70**, 2614.

[5] Wright, L. D., Skeggs, H. R., and Huff, J. W., *J. Biol. Chem.*, 1948, **175**, 475.

[6] Short, M. S., *J. Bact.*, 1947, **53**, 669.

[7] Lewis, U. J., Register, U. D., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1949, **71**, 509.

We are indebted to Merck and Co., Inc., Rahway, N. J., for crystalline vitamins, and to Dr. B. L. Hutchings of the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y., for synthetic folic acid.

Received August 29, 1949. P.S.E.B.M., 1949, **72**.

Observations on Diaphragm and Stomach of the Dog Following Phrenicotomy.* (17476)

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In a recent communication we have pointed out some of the relationships between the left diaphragm and gastro-intestinal mechanisms in the dog.[1] The significant feature of these observations was a decrease in gastric emptying time in the presence of a relatively dilated stomach after left sided transthoracic phrenicotomy. The dogs were kept for prolonged periods in order to observe whether the denervated diaphragm would resume its respiratory function. This was done in view of Noack's work, who reported that regular rhythmic respiratory contractions of the diaphragm reappeared in the human from one and a half to two years after phrenic exeresis in the neck.[2] Noack's statement has been accepted by a number of authors. The mechanism of recovery of diaphragmatic function was postulated to be through the twelfth thoracic intercostal nerves and through sympathetic innervation. If the innervation of the diaphragm and recovery of its function following interruption of the phrenic nerve in man and dog are comparable, experiments on the latter offer the advantage of better control of most experimental conditions and ensure a complete section of the phrenic nerve, which is not always possible in the usual operations in the neck of man for phrenic exeresis. Long term observations comparable to those done by Noack on the human were done on the dog. The animals were observed fluoroscopically, in order to determine if and when there would occur reactivation of the diaphragm after complete left transthoracic phrenic section, the right diaphragm serving as normal control. Sauerbruch[3] described a gastrocardiac symptom complex following left side phrenic exeresis, which was later called Roemheld's syndrome. The symptoms were bradycardia, premature systoles, anxiety, epigastric distress, lack of appetite, pallor, nausea, vomiting and eructations.

Experimental. Sixteen dogs were operated upon transthoracically under aseptic conditions, using pentobarbital sodium anesthesia. In 13 animals a section of the left phrenic nerve from the hilum of the lung to the diaphragm was removed. Two dogs had simple phrenic section at the level of the diaphragm, and one had the nerve cut at the hilum of the lung. The cut ends of the nerve were doubled up and tied with non-absorbable suture. The dogs appeared normal after a few days of postoperative care. All dogs were fluoroscoped at weekly intervals for the first 12 to 14 weeks after operation and then at monthly intervals, to watch for possible reactivation of the diaphragm and for the possible development of Roemheld's syn-

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* Aided by a grant from the Otto Baer Fund.

The Department is in part supported by the Michael Reese Research Foundation.

[1] Jefferson, Nelson C., and Necheles, H., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 166.

[2] Noack, R., *Beitr. Z. Klin d. Tuberk.*, 1933, **82**, 397.

[3] Quoted by Roemheld, L., *Münch. Med. Wchnschr.*, 1928, **75**, 1872.

drome. Observations were made also after standard barium meals[4] to determine if there was a relationhsip between the possible reactivation of the diaphragm and the return to normal of the gastric emptying time. Eight of the dogs were observed for periods of 14, 15, 16, 18, 19, 19, 20 and 20 months postoperatively, at which time acute experiments were done under pentobarbital sodium anesthesia and with artificial respiration, to determine whether impulses would reach the diaphragm upon stimulation of the phrenic nerve above the point of the previous section. In these experiments, gastric motility and blood pressure recordings were made before and after stimulation of the phrenic nerve with various strengths of induced current from a Harvard inductorium. At the termination of all experiments sections were made from the right and left diaphragm and from the proximal and distal phrenic nerve stumps for microscopic study.

Results. Using fluoroscopy, we have not seen in any of 15 dogs a completely denervated diaphragm reactivate during 20 months of observations. The shortened gastric emptying time returned to normal in periods varying from several weeks to several months. The large gas bubble within a moderately dilated stomach however persisted for many months. At no time was respiratory difficulty observed, but the dogs were not subjected to severe exercise. Acute experiments on 8 dogs 14, 15, 16, 18, 19, 19, 20 and 20 months after phrenic section revealed a fixed, elevated, atrophic left diaphragm more fixed by intraabdominal pressure than by adhesions. The muscle was paper-thin and translucent. In one dog (19 months) there was a completely atrophied anterio-lateral one half of the left diaphragm and a normal posterio-lateral half. Upon inspection of the opened chest under anesthesia, the normal part showed rhythmic contractions, while the atrophied part did not. However careful dissection and inspection revealed one intact accessory phrenic nerve, which had not been cut. In this animal, fluoroscopy had been difficult, and we were not certain whether the diaphragm moved or not.

Sections[†] from the left diaphragm revealed degeneration and disappearance of nerve fibers and degenerated muscle, replaced by fibrous tissue and fat. Control sections from the right diaphragm were normal. The phrenic nerve above the point of section revealed degenerating, regenerating, or normal nerve fibers. The phrenic nerve distal to the section revealed complete degeneration. Stimulation of the distal or proximal ends of the cut phrenic nerve did not reveal any effect on the diaphragm in the 7 dogs with complete phrenicotomy, which were tested in acute experiments. Stimulation of the proximal phrenic stump caused a rise in blood pressure and, in some experiments, increased respiration. When the stump was cut central to the electrode these reflexes were abolished. The gastro-cardiac symptom complex of Roemheld was never observed.

Discussion. Credit for first interrupting the phrenic nerve in man is given to Stuertz[5] and to Sauerbruch.[6] The exhaustive study of Felix[7] established a basis for the operation. He found that the phrenic nerve receives sympathetic fibers in the neck and at the level of the diaphragm, as well as spinal fibers from lower cervical and first thoracic nerves. The phrenic does not give branches to the pericardium. The left phrenic divides into an anterior, lateral and posterior branch to supply the diaphragm. The importance of an accessory phrenic is stressed, which may be easily missed by the surgeon, and which will allow for continued phrenic impulses to the diaphragm. He concludes that the motor innervation of the diaphragm consists of the phrenic, the 12th intercostal nerve, and probably sympathetic fibers. The phrenic supplies the main part of the diaphragm, the 12th intercostal the root of the diaphragm from the 12th rib, and sympathetic and phrenic together supply the lumbar section of the diaphragm. Schwatt[8] studied 138 cases after phrenic exeresis or/and phrenicotomy and observed that, during the earlier period of from two to eighteen months after the operation, paradoxical movements of the diaphragm were the rule. Ultimately however, probably because of degeneration and atrophy of the diaphragmatic musculature plus pleural adhesions, fixation occurred. Davies[9] lists 3 operations for paralyzing the diaphragm, namely: phrenic avulsion, phrenicotomy, and phrenic crush. "The immediate effect of these operations is paralysis of the hemidiaphragm; the muscle fibers degenerate and the dome becomes a sheet of fibrous tissue within approximately four months. As a result of the paralysis there are three changes of the dome, namely: a loss of tone, a rise into the thorax, and a cessation of respiratory movements." Klein[10] states that many failures to secure paralysis of the hemidiaphragm are due to the presence of accessory branches of the phrenic nerve, which join the main trunk deep in the thorax, and which can carry on the function of the phrenic nerve if they are not cut. Twenty per cent to thirty per cent of all individuals are supposed to have accessory branches. Geraghty and Aycock[11] state that the length of phrenic nerve avulsed is of importance, because of the possibility of return of diaphragmatic function unless all auxiliary branches are interrupted. In their series the maximum avulsed length was 30 cm., and the minimum 2.5 cm. Matson[12] states that an avulsion of not less than 10 cm means a return of diaphragmatic function in 25 to 30% of all patients. Schwatt[13] concludes that an avulsion of 10 to 12 cm is necessary, as this approximates the distance from the operative site to the hilum and was sufficient to interrupt all accessory fibers. Anderson[14] states that the diaphragm may continue to rise for 6 months and that the immediate rise is by no means the maximum. Aufses,[15] in a review of the anatomy of the phrenic nerves, states that most of the accessories are not too intimately connected with blood vessels and join the main trunk within a distance of 11 to 13 cm from the level at which it crosses the scalenus anticus. Therefore, if avulsion of the nerve is performed, and at least 13 cm are removed, one may be confident that a total permanent paralysis of the hemidiaphragm will ensue. Further he states that the phrenic nerve is the sole motor nerve to its corresponding hemidiaphragm, and that interruption causes immediate paralysis of the muscle with atrophy which begins in a few weeks and progresses until it becomes complete at about the third or fourth month.

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[4] Kozoll, D. D., and Necheles, H., *Surg.*, 1942, **2**, 360.

[†] We acknowledge the help of Dr. O. Saphir, Pathologist of Michael Reese Hospital.

[5] Stuertz, *Deutsche Med. Wchnschr.*, 1911, **37**, 2224, quoted by Felix.[7]

[6] Sauerbruch, F., *Chirurgie der Brustorgane*, ed. 3, II, Julius Springer, Berlin, 1930.

[7] Felix, W., *Deutsche Z. f. Chir.*, 1922, **171**, 283.

Aree[16] states that the phrenic nerve has anastomoses with the sympathetic system when it enters the thorax, and that some of the branches of the phrenic are distributed to the pulmonary hilum. The latter fact makes it possible that phrenic section may produce a decrease of pulmonary reflexes. Textbook information assures that in the human, following crushing of the phrenic nerve, the diaphragm reactivates in about 3 months.[17] According to Noack,[2] after exeresis in the neck of 10 to 30 cm of phrenic nerve, the diaphragm reactivated in from 1½ to 2 years, and he attributes this to fibers of the twelfth intercostal nerve and to sympathetic innervation. Christopher[17] states that for permanent paralysis not less than 4½ to 5 inches of the main trunk of the phrenic nerve must be avulsed from within the thorax. This will usually sever accessory branches (present in 20% of cases) which join the main nerve along its course to the diaphragm. Occasionally one or more accessory nerves may have an independent course to the diaphragm. Therefore avulsion of the main nerve may or may not interrupt all accessory branches of the phrenic nerve. Since after 20 months we have found only atrophy of the denervated diaphragm in our dogs it would appear that either the innervation is not the same in the dog as in the human, or that our procedure on the dog involved complete section, while in man, where the procedure is done in the neck, all phrenic fibers may or may not be avulsed, no matter how much of the main nerve is removed. No emphasis seems to be placed by the many writers on tying the cut ends of the nerve with non-absorbable suture, a feature which to us appears to be of some importance. We have found that the phrenic nerve is able to regenerate within a period of 20 months.‡ Since the musculature of the diaphragm in both species consists of striated muscle, and the phrenic is a somatic nerve, it follows that, once true paralysis and atrophy has developed in the muscle it would never be capable of reactivation. The so-called paralysis as seen in the human is probably in many instances palsy and not paralysis, as outlined by Christopher.[17]

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[8] Schwatt, H., *Am. J. M. Sc.*, 1934, **187**, 338.

[9] Davies, H. M., *The Lancet*, 1935, **229**, 418.

[10] Klein, B., *Illinois Med. J.*, 1938, **73**, 418.

[11] Geraghty, F. J., and Aycock, T. B., *The West Virginia Med. J.*, 1940, **36**, 546.

[12] Matson, R. W., *Am. Rev. Tuber.*, 1930, **22**, 1.

[13] Schwatt, H., *J. Thor. Surg.*, 1934, **3**, 503.

[14] Anderson, B. W., *Edinburgh Med. J.*, 1934, **41**, 169.

[15] Aufses, A. H., *Am. J. Surg.*, 1940, **50**, 715.

[16] Aree, J., and Breu, M. M., *J. Thor. Surg.*, 1943, **12**, 544.

[17] Christopher, F., A Textbook of Surgery, 3rd ed., W. B. Saunders Co., Philadelphia and London, 1943.

The fact that we obtained complete paralysis and not palsy may be speculated upon. Our operations were transthoracic, and all observable fibers were cut and tied with a non-absorbable suture. The one instance observed by us in which partial diaphragmatic function persisted and in which complete atrophy of one half of the left diaphragm was found 19 months after supposed complete section of the phrenic nerve, was demonstrated to be due to an intact accessory phrenic nerve missed at operation. This would of course indicate that once atrophy, always atrophy, and that in the human so-called reactivation might have been confused with such a picture as we observed in this animal.

The reason for the absence of Roemheld's syndrome in our dogs may lie in the explanation given by Noack,[2] namely, that the tearing of sympathetic fibers during phrenic avulsion in the neck and not section of the phrenic nerve itself leads to the syndrome.

Paradoxical movements of the diaphragm following phrenicotomy, as described by Schwatt[8] were not observed in our experiments.

Summary. 1. No reactivation of the left diaphragm was seen by us in 15 dogs up to 20 months after complete section of the left phrenic nerve.

2. Microscopically all diaphragms showed complete muscular atrophy with degeneration or disappearance of nerve fibers, 14 to 20 months after complete section of the left phrenic nerve. It is difficult to conceive how such atrophic muscles could ever regenerate.

3. It is highly possible that phrenic exeresis in the neck of the human does not always result in complete phrenicotomy with subsequent atrophy of the diaphragm, but in a number of instances results in diaphragmatic palsy.

4. We found no evidence that the twelfth thoracic intercostal nerve or sympathetic innervation could influence the reactivation of the diaphragm in the dog.

5. Our experience with one diaphragm leads us to believe that a hemifunction of one half of a diaphragm may lead the fluoroscopist to assume that complete reactivation of that diaphragm has occurred.

6. The phrenic nerve above the point of section was able to convey reflexes 20 months after section; this was demonstrated by rise in blood pressure and increased respiration upon stimulation of the central end of the nerve, which were abolished when the nerve was cut central to the stimulating electrode.

7. No evidence of the gastro-cardiac symptom-complex of Roemheld was seen.

Received October 25, 1949. P.S.E.B.M., 1949, **72**.

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‡ Unpublished work.

The Coenzyme A Content of Ischemic Dog Myocardium. (17477)

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We have previously reported the destruction of coenzyme I in dog myocardium rendered ischemic by ligation of the left anterior descending coronary artery. In similar experiments cocarboxylase was found unchanged after ischemia.[1] In the case of coenzyme I, the destruction described could be prevented by pretreatment with large doses of nicotinamide.

It may be considered that the occurrence of breakdown of a coenzyme under these conditions can suggest the likelihood of favorable results in treatment of coronary occlusion with large doses of the vitamin corresponding to the coenzyme concerned. On this basis it was thought of interest to determine whether or not coenzyme A is broken down in ischemic myocardium and whether the breakdown, if any, may be prevented by pantothenic acid administration.

Methods. The left anterior descending coronary artery was ligated in ten dogs by essentially the same technique reported previously. Ischemic and control myocardial samples were removed after 2 hours, boiled, homogenized, and analyzed for coenzyme A by the method of Kaplan and Lipmann.[2] Four of the 10 dogs were pretreated with 50 mg/kg of sodium pantothenate intravenously 10 minutes before ligation of the vessel.

Results. The accompanying table shows in Kaplan-Lipmann units per gram dry muscle, the myocardial content of coenzyme A together with the percent change after 2 hours of ischemia. It is evident that breakdown of coenzyme A does occur, although by no means to the extent that coenzyme I is degraded, and that pantothenate does not significantly relieve the loss of coenzyme. This could suggest that the breakdown is not enzymically catalyzed, that heart muscle does not synthesize coenzyme A, or that pantothenic acid needs molecular rearrangement before being available for coenzyme A synthesis by myocardium.

Summary. Acute coronary artery ligation in the dog results in loss of coenzyme A in the infarcted myocardial area. Pretreatment with pantothenic acid does not influence this loss.

Received October 25, 1949. P.S.E.B.M., 1949, **72**.

TABLE I.
Myocardial Coenzyme A Content in Lipmann Units/G Dry Wt.

Not pretreated				Pretreated			
Dog	Normal	Ischemic	% change	Dog	Normal	Ischemic	% change
A	166	53	—68	G	227	111	—53
B	173	106	—39	H	184	162	—12
C	204	155	—24	J	168	143	—15
D	166	145	—13	K	125	107	—14
E	176	155	—12				
F	145	90	—38				

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[1] Govier, Wm. M., *Am. Heart J.*, 1945, **29**, 384.

[2] Kaplan, Nathan O., and Lipmann, Fritz, *J. Biol. Chem.*, 1948, **174**, 37.

Effect of Protein Depletion upon Susceptibility of Rats to Total Body Irradiation. (17478)

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Although there have been many studies of the effects of penetrating radiation on normal animals of various species, there has been little investigation of the effects of radiation upon abnormal animals. Some components of diet, particularly the vitamin B series[1] and some of the amino acids,[2] have been reported to alter the response to radiation but the broader problems of protein, fat, or carbohydrate nutrition have not, to our knowledge, been investigated to any great extent. Since widespread tissue necrosis is a prominent feature of radiation damage, it appeared that investigation of the effect of protein, particularly a deficiency of protein, on the response to radiant energy might prove instructive. Therefore a series of experiments, of which this is the first report, was undertaken to try to evaluate the role of protein in radiation damage.

Procedure. This experiment was planned to determine the effect of moderately severe depletion of the protein reserves on the susceptibility to radiation. Since young adult rats showing 25% reduction in weight while on a protein-poor diet are known to have but little reserve protein,[3] this type of animal was selected for this study. There is considerable confusion regarding the effect of age and weight on susceptibility to radiation. For this reason two types of control animals were used, one group having the same weight as the test animals, the other being the same age. A total of 141 rats was almost equally divided into the 3 groups in this experiment. Protein-depletion, as it is produced by the method developed by Cannon and his group, consists of placing the rats on a low protein diet that is adequate in calories, vitamins, and minerals. The diets and methods used in obtaining protein-depleted rats have been described elsewhere by Cannon[4] and Wissler.[5] In this experiment male albino rats of the Sprague-Dawley strain were used. Rats with an initial weight of about 200 g were placed on a diet (3E) containing 1.9% protein. The animals were kept in large, wire-bottom cages with 6 rats to a cage. The rats were continued on the depletion diet until they had lost 25% of their starting weight. Such a weight loss occurred in about 10 weeks. The animals were then given total body irradiation.

Total body irradiation of all of the animals was accomplished by x-ray. The radiation was done at 200 kv and 15 ma with 0.5 mm copper and 3 mm bakelite filters. The half-value layer of the radiation was 1.2 mm of copper. The target to animals distance was 71 cm and the dose rate in all cases was 17-18 r per minute. The dosage rate was measured before and after each exposure with a Victoreen electrometer. The animals were placed in individual perforated aluminum cages and radiated in a horizontal beam. The cages were regrouped and turned several times during the exposure to give the animals uniform distribution in the beam.

The two groups of control rats were maintained on a diet (3C) identical with the protein-deficient ration except that it contained 22% protein supplied by casein. The age control rats were placed on this diet after they had reached a starting weight of about 200 grams. The animals were continued on this program until they had been on the 22% protein diet for a period of time equal to the period of depletion for the protein-depleted rats. At time of radiation these animals were 20 weeks old and weighed about 280 g. For the second control group, rats with an initial weight of about 100 g were fed the 22% protein ration at a rate of 15 g per rat per day until they had reached a weight of approximately 150 g. These rats were then about 8 weeks old and were given total body irradiation. Three groups of these rats were radiated before they had reached a weight of 150 g. The results show that this did not lessen their value as control animals.

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* Atomic Energy Commission Postdoctoral Research Fellow in Medical Sciences of the National Research Council.

[1] Martin, C. L., Moursand, W. H., *Radiology*, 1938, **30**, 277.

[2] Patt, H. M., Tyree, E. B., Straube, R. L., Smith, D. E., *Science*, 1949, **110**, 213.

[3] Benditt, E. P., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 243.

[4] Cannon, P. R., Wissler, R. W., Woolridge, R. L., and Benditt, E. P., *Ann. Surg.*, 1944, **120**, 514.

[5] Wissler, R. W., Woolridge, R. L., Steffee, H. S., and Cannon, P. R., *J. Immun.*, 1946, **52**, 267.

During the 30-day observation period following radiation all of the animals were continued on the same diets they had been receiving prior to radiation. All of the rats were weighed at 2-day intervals through this observation period.

These experiments were performed over a 9-months period. For the most part corresponding groups of depleted and age control animals were radiated at the same time and these groups were under observation at the same time with similar animal colony conditions. It was not feasible to radiate weight control animals at the same time as the other groups. Nevertheless groups of all 3 types of rats were scattered through the 9-months period in an attempt to keep the conditions in each series as nearly comparable as possible.

Results. The results of this experiment are shown in Table I. Here it is apparent that the mortality in the protein-depleted animals was considerably greater at any given dosage than in the two control groups. Most of the deaths in the protein-depleted groups of rats occurred below 625 r while there were few deaths in the control groups below this dose.

In order to plot definite dosage-mortality relationships, the data were treated in the manner developed by Bliss.[6,7] The regression lines derived from this procedure are shown in Fig. 1. This treatment of the data demonstrates clearly that the protein-depleted rats were considerably more susceptible to total-body irradiation than were either animals of the same age or of the same weight when fed adequate protein in their diet.

TABLE I.
Mortality in Rats Following Total Body Irradiation.

Dose	No. animals	Avg wt, g	Died
	Depleted rats		
300r	4	158	1
450	6	160	0
525	6	162	1
525	3	154	2
575	6	146	2
575	3	155	3
600	6	156	6
625	3	150	2
625	3	161	3
750	6	157	5
	Age controls		
525r	3	286	0
575	6	293	0
625	3	304	0
650	3	297	1
675	9	268	6
700	3	299	1
725	9	269	6
750	3	299	2
775	9	269	8
800	2	291	2
	Wt controls		
500r	5	155	0
600	5	153	2
600	10	128	1
650	10	128	3
700	5	156	4
700	10	128	4

The equation for the regression line for the protein-depleted animals is: $Y = 5.3460 + 8.9179\ (X - 2.7463)$ where Y equals the probit value of the mortality and X is the logarithm of the observed dose. The standard deviation for this line is .1121. The regression line of the animals of the same age is: $Y = 5.1545 + 21.0772\ (X - 2.8476)$ with a standard deviation of .0474. Rats of the same weight give: $Y = 4.7268 + 15.0372\ (X - 2.8135)$ and a standard deviation of .0665. The fiducial ranges, or confidence limits of the dosage, with a probability of .05 for the regression lines at the lethal dose for 50% of the animals in 30 days (LD 50/30) were calculated according to Bliss[8]

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[6] Bliss, C. I., *Ann. Applied Biol.*, 1935, **22**, 134.

[7] *Ibid.*, 1935, **22**, 307.

[8] Bliss, C. I., *Quart. J. and Yearbook Pharmacy*, 1938, **11**, 192.

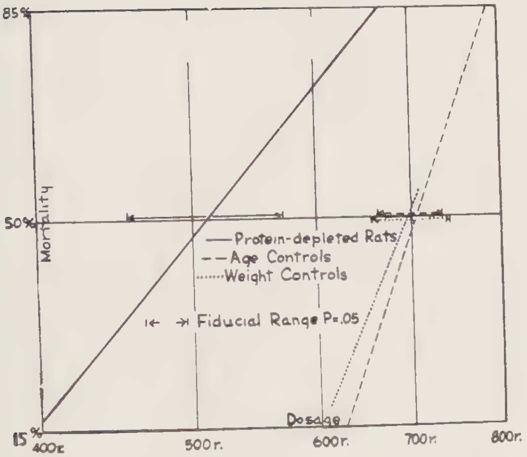

Fig. 1.

Thirty-day dosage-mortality regression lines in rats following total body irradiation.

and are indicated by corresponding arrows in Fig. 1. As can be seen, there is a wide zone separating the limits of variation of the protein-depleted animals from the limits of the controls.

In this experiment the weights of the protein-depleted radiated rats that survived the 30-day observation period closely paralleled the weight loss in non-radiated depleted animals except for some additional weight loss in the period immediately following radiation. Both the age control and the weight control rats also showed weight loss in the first few days following radiation when diet consumption was poor. This loss was quickly recovered when these animals regained their appetite and they continued to gain weight through the remainder of the observation period. It is important to note that a period of 100 days on the protein-deficient diet, as was the case with these depleted animals, causes no mortality in non-radiated rats. Rats have been kept on a protein-deficient diet for as long as 6 months in this laboratory with little spontaneous mortality, providing care is taken to prevent any infectious diseases of rats. Thus it is not likely that any of the increased mortality observed in the protein-depleted rats was due to spontaneous mortality.

In all groups of radiated animals, as is the case in most radiation mortality studies, death usually occurred before the fifteenth post-irradiation day. Autopsies were done on most of the rats that died and no differences were noted between the lesions in the control and depleted rats. Most of the animals that died in the first few days following radiation showed widespread interstitial hemorrhages. Some of the rats that died later showed pneumonia. Blood cultures were done on 12 of the protein-depleted rats that died after exposure of less than 650r. Two organisms were found consistently in the 10 animals that died before the fifteenth post-irradiation day. One organism was a gram-negative rod (probably *Bacillus dysenteriae*) and the other a micrococcus. The 2 depleted animals that died after the fifteenth day both had sterile blood cultures.

Discussion. The present experiment shows that the protein-depleted rat is materially more susceptible to the physical injury of penetrating radiation than is the animal on an adequate protein diet. The LD 50/30 for the protein-depleted rat is approximately 520r while the LD 50/30 for the control rats in this series proved to be in the region of 700r. Thus at the LD 50/30 there is a great difference in the dosage between depleted and control rats. This increased susceptibility of the protein-depleted rat becomes even more apparent when one realizes that the dose of total body irradiation that killed 50% of the protein-depleted rats killed less than two per cent of the control rats. The slope of the regression line for the protein-depleted animals indicates that these animals are proportionately somewhat more susceptible to lower doses of radiation than the controls.

The fiducial range for each of the regression lines, as shown in Fig. 1, shows a wide zone separating these ranges at the LD 50/30. Calculation of the differences at the LD 50/30 indicates that the P value for the data at this point is somewhat less than .001. Therefore the observed increase in mortality from radiation in the protein-depleted rat is highly significant.

Since there is confusion about the influence of weight and age on radiation susceptibility, control rats for both of these factors were used. Quastler[9] found that in mice, with doses in the range of the LD 50, the survival time was proportional to body weight and age. Hagen and Sacher[10] and Hagen and co-workers[11] reported that in a series of mortality studies on rabbits the animals weighing more than 2 kg were less sensitive to x-ray than rabbits weighing less than 2 kg. Naiman[12] recently reported that older, and therefore heavier, rats could sustain larger doses of radiation than small rats. In contrast to these conclusions Hagen and Simmons[13] studied a series of 638 rats of the same strain as that used in this experiment and concluded that "within the range tested (median weight 210 g) any age or weight effect on radioresistance of rats must be small." Boche and Bishop in a study of 240 rats of the Wistar strain concluded that age and sex did not cause significant differences in radiation mortality.[14] The coincidence of the two control regression lines in this experiment and the corresponding fiducial ranges indicate that age and weight in the adult rat, in the range of 150 to 280 g, have little effect on susceptibility to radiation under the conditions of this study.

The LD 50/30 of about 700r for the 2 groups of control rats in this experiment is in close agreement with the LD 50/30 being obtained at present for similar animals in another laboratory.[15] This figure is in some disagreement with a LD 50/30 of 600r as reported by Prosser[16] but is in only moderate disagreement with a LD 50/30 of 640 ± 5r for a Wistar strain of rats as recently reported by Clark and Uncapher.[17]

The finding of increased susceptibility to radiation damage in protein-depletion coincides with findings of increased susceptibility in protein-depleted rats to other types of injury. The studies of Cannon,[4,18] Wissler[5,19] and Benditt[3] have demonstrated that antibody production is seriously impaired by severe and prolonged protein-depletion. Taliaferro and associates[20] recently demonstrated a decreased immunity to trichinosis in the protein-depleted rat. A delay in wound healing was noted by Kobak and associates[21] in protein-depletion. The leukocytic response of the bone marrow, particularly to repeated stimulation, is also markedly impaired by depletion of protein reserves.[22]

Protein-depletion, in addition to causing some decrease in plasma proteins and hemoglobin level,[23] also causes a decrease in production of several of the enzymes. Tobin and co-workers[24] have shown a reduction in hyaluronidase and antihyaluronidase. Benditt and associates[25] have demonstrated that cytochrome oxidase and succinoxidase are decreased in liver homogenates in protein-depletion. Kidney phosphatase is also reduced while the total liver phosphatase remains constant during the depletion of the protein reserves. It is known that radiation also inhibits several of the enzymes, particularly the sulfhydryl-containing enzymes, as shown by the recent work of Barron and his group.[26]

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The manner in which protein-depletion increases susceptibility to radiation is not known. Several methods by which radiation may cause death have been postulated and protein-depletion affects most of these mechanisms. Death following radiation may be due to infection subsequent to the break-down of the normal immunological processes. It is well known that protein-depletion interferes with immunological responses and this in combination with the effects of radiation on the epithelial barriers, particularly the intestinal epithelium, may account for the increased susceptibility. Also it may be more difficult for the depleted animals to recover from the leukopenia of radiation so that the phagocytic barrier against infection is lowered. There is much evidence that the degree and duration of leukopenia following radiation have great influence on mortality.[27]

Since it is known that protein-depletion and radiation both interfere with enzyme production or action, it may be that there is an additive effect that interferes with enzyme activity to account for the increased radiation susceptibility.

It may be that death is due to anemia following bone marrow damage. That this mechanism accounts for the increased susceptibility does not seem likely for protein-depletion *per se* does not cause a marked lowering in the erythrocyte or hemoglobin levels. However it may interfere with the recovery of the bone marrow and with the production of both erythrocytes and leukocytes.

It seems possible that the increased susceptibility to radiation observed in these protein-depleted animals might be due to their poor immunological and leukocytic responses or to their decreased production of some essential enzyme. This problem is being investigated further.

Conclusions. 1. Protein-depletion in young adult male rats, as produced by a weight loss of 25% on a protein-poor diet, materially increases susceptibility to total body irradiation. 2. Age or weight in normally nourished adult rats, in the 150 to 280 g weight range, have little effect on susceptibility to radiation.

The author wishes to express his appreciation for advice and assistance to the following men: P. R. Cannon, R. W. Wissler, and E. P. Benditt, Department of Pathology, University of Chicago; A. M. Brues, Biology Division, Argonne National Laboratory, Chicago; L. J. Savage, Department of Mathematics, University of Chicago; and H. D. Landahl, Committee on Mathematical Biology, University of Chicago.

The animals used in this experiment were irradiated through the collaboration of the Biology Division, Argonne National Laboratory Chicago.

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Received October 25, 1949. P.S.E.B.M., 1949, **72**.

An Autohemolytic Agent in Fetal Liver Extracts.* (17479)

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In the course of studies dealing with enzymatic activity during fetal and extrauterine development it was noted that extracts of fetal liver lysed the red cells of the same species. It was of interest to determine the nature of this phenomenon and to compare the activity of adult and fetal extracts.

Methods. Thirty experiments were performed, mostly on pregnant guinea pigs and their fetuses. A few experiments were made with pregnant rats or newborn rats and their mothers. The pregnant animals were killed by decapitation and the maternal blood was collected in oxalated tubes. The fetuses were then quickly removed and decapitated and

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* This investigation was supported in part by a grant from the National Cancer Institute, U. S. Public Health Service.

the blood collected in separate oxalated tubes. The fetal and maternal livers, spleens, kidneys, hearts and lungs were perfused *in situ* to wash out as much blood as possible and then removed and frozen on dry ice. When all the tissues were removed and frozen they were weighed, then ground in a porcelain mortar and 0.9% NaCl was added to obtain suspensions varying from 5 to 30%. These suspensions were centrifuged and the supernatant fluid was used in subsequent tests.

In the experiments reported here the activity of each extract was tested on both fetal and maternal red cells. In several runs a suspension of red cells was used from a species other than the one from which the tissue extracts were prepared. In all instances, the cells were washed three times with 10 to 12 volumes of physiological saline and used in the test as a 1% suspension.

The supernatant fluid of the tissue suspension was diluted by pipetting into several 12 x 75 mm test tubes 1.0, 0.5, 0.25 and 0.10 ml of the extract and making them up to 1.0 ml with 0.9% saline. Several tubes containing 1.0 ml saline were prepared to serve as controls. One ml of the red cell suspension was then added to these tubes. Since some fetal liver extracts, despite *in situ* perfusion, possessed interfering color it was necessary to set up a series of tubes containing various dilutions of the extract together with 1.0 ml of saline instead of the red cell suspensions. These tubes served as color controls. The tubes were placed in an incubator (38°C) and at one-half hour intervals they were shaken. After 2½ to 4 hours incubation they were removed and centrifuged at 2,000 RPM for 5 minutes. The degree of hemolysis was determined visually by the amount of color in the supernatant liquid (corrected for by the "color controls") and by the amount of sediment. The usual grades, 4, indicating complete hemolysis, to 0, for none, were employed.

Results. As illustrated in Table I, extracts of fetal guinea pig liver (crown-rump size from 6 to 9 cm) lysed *in vitro,* and within 4 hours, suspensions of red cells from the blood of the same fetus, as well as from that of the mother. They also exhibited this lytic action on red cells from the male of the same species, as well as against a red cell suspension of rat blood. This activity could be demonstrated in dilutions equivalent to 0.5% of the original suspensions. Extracts of maternal guinea pig liver exhibited detectable activity only in relatively high concentrations and, in the experiments reported here, failed to lyse within the 4 hour period the red cells of the rat. Extracts of other fetal tissues (Table II), with the possible exception of the spleen, did not show as high activity as that found for liver, nor were the fetal-maternal differences as consistent as those found for liver. Extracts of fetal guinea pig spleen, in 2 experiments, and fetal kidney, in one experiment, possessed greater lytic activity than the corresponding maternal extracts. In the rat, fetal liver and spleen possessed greater activity than the maternal organs. Extracts of adult rat lung and adult guinea pig lung appeared to produce more lysis than the corresponding fetal organs. However, in these experiments very little lysis was produced after 4 hours.

Boiling the extract of fetal guinea pig liver for one minute completely destroyed the activity (Table I.). The precipitate of such a heated extract caused a small amount of hemolysis at the end of 4 hours, indicating that a heat-stable substance was carried down by the precipitate. Heating for one hour at 56°C destroyed about 75% of the activity. The addition of fresh serum to such a heat-inactivated extract did not restore the activity. Apparently complement, in the classical sense, is not involved in the hemolysis. Allowing the extracts to stand for one hour with heated (to 56°C) or fresh serum before the red cells are added reduces the amount of hemolysis. This may mean that an anti-hemolytic factor that is not a protein is present in the serum.

If the complete system (extracts plus red cell suspension) is allowed to stand overnight, a marked increase in hemolysis is observed and, in some experiments with liver extracts, the fetal-maternal differences are obscured.

TABLE I.
Hemolytic Action of Saline Extracts of Fetal and Maternal Guinea Pig Livers.

	Dilution (see text)			
	15.0	7.5	3.8	1.5
A. Against maternal red cells				
1. Unheated				
Fetal	++++	++++	++	±
Maternal	+	±	0	0
2. Boiled 1 min.				
Fetal	0	0	0	0
Maternal	0	0	0	0
3. Ppt. of boiled extr.				
Fetal	+	±	0	0
Maternal	0	0	0	0
4. Heated to 56°C 1 hr				
Fetal	++	++	0	0
Maternal	0	0	0	0
5. Effect of 0.5 cc serum (See text)				
Fetal	+++	++	±	0
Maternal	0	0	0	0
	20.0	10.0	5.0	2.5
B. Against fetal red cells				
Fetal	++++	+++	++	0
Maternal	±	0	0	0
	15.0	7.5	3.8	1.5
C. Against rat red cells				
Fetal	++++	+++	++	0
Maternal	0	0	0	0

If the extracts alone are allowed to stand for 12 hours and then tested, a marked increase in their ability to produce lysis is found. On the other hand, the addition of the sodium salt of monoiodoacetic acid to the extracts in a final concentration of 0.003 M inhibits the increase of activity due to standing.

Discussion. The results indicate that saline extracts of fetal guinea pig liver possess greater lytic activity than similarly prepared adult (maternal) extracts. The activity is due to both heat-labile and heat-resistant factors but the heat-sensitive agent accounts for the greater part of the lysis under the conditions of these experiments. At present, we cannot account for the fetal-maternal difference reported here. There is, however, a considerable body of literature that may give us insight into the probable nature of some of the lytic agents or systems involved.

There is substantial support for the idea that at least 3 hemolytic systems involving lysins exist *in vivo:* (1) It is well known that the spleen plays an important role in preparing red cells for destruction. Rous,[1] Krumbhaar,[2] Dameshek,[3,4] and Ponder[5] have reviewed the evidence bearing on this. It has been suggested that the agent responsible for this action of the spleen is produced by enzymatic action and the resulting substance has been identified as lysolecithin.[6,7,8] This

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TABLE II.

Hemolytic Action of Saline Extracts of Other Tissues Using Maternal Red Cell Suspensions.

	Dilution (see text)			
	15.0	7.5	3.8	1.5
A. Guinea pig				
1. Spleen				
Fetal	++	+	0	0
Maternal	+	+	0	0
2. Kidney				
Fetal	+	±	0	0
Maternal	±	0	0	0
3. Lung				
Fetal	0	0	0	0
Maternal	++	++	+	+
B. Rat				
1. Liver				
Fetal	++++	+	±	0
Maternal	++	0	0	0
2. Spleen				
Fetal	++++	++++	+++	+
Maternal	±	±	0	0
3. Lung (Homogenized in blendor)				
Fetal	++	++	+	±
Maternal	+++	+++	++	+

system is heat-sensitive and is inactivated at 56°C. (2) That lysins can be obtained from tissues other than the spleen has been shown by Metchnikoff,[9] Weil,[10] Maegraith, Findlay and Martin,[11] and Ponder.[12] Weil found that kidney and liver extracts contained lysins and that their activity was inhibited by serum. He also pointed out that the lytic activity of some extracts may be derived from residual blood in the tissue. In the experiments reported here it was extremely difficult to obtain blood-free saline extracts of fetal liver. However, it should be pointed out that the lytic action of fetal liver was equally as potent against the red cells of the same fetus as against the maternal or rat blood cells. Maegraith, Findlay and Martin[11] have recently described a heat-sensitive agent found in a variety of human and animal tissues. This agent produces lysis after 24 hours of incubation at 37°C. Their lysin is inactivated at 80°C in 5 minutes and they report it to be species-specific. Ponder[12] has been able to confirm in part the observations of Maegraith, *et al.*, but he finds that extracts he prepared are not species-specific. Weil[10] did not determine whether his extracts lysed the red cells of other species. The heat-sensitivity and lack of species-specificity suggest that the predominant factor in the fetal liver is similar, as far as those two characteristics are concerned, to the agent found by Ponder,[12] but differ as to the time required to produce lysis. This may be due to a higher concentration of the factor in fetal liver. The results also suggest that it may be lysolecithin, or some closely related substance. The inhibitory action of iodoacetate in preventing the accumulation of such lytic agents might be accounted for by its activity on the enzyme responsible for the production of the lysolecithin.

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Although heat-labile factors are responsible for the greater portion of the activity reported in these experiments a small amount is found to be due to heat-resistant substances. This brings up the question of their probable nature.

(3) In addition to the two hemolytic systems briefly described above there is evidence for the existence of a third system. Freeman and Johnson[13] and Loewy, *et al.*[14] have shown that an increase in soaps in chyle, particularly during fat absorption after a meal high in fats, can be large enough to bring about a degree of hemolysis on entering the blood. The fetal guinea pig liver, at the stages studied, has a very high fat content[15] and it is conceivable that as a result there is a sufficient increase in the soap content to account for the heat-stable hemolytic agent.

The fact that serum heated, as well as fresh, inhibits the activity of the lytic agents points to the probability that this may be part of an *in vivo* counteracting mechanism.

Bearing on the general phenomena reported here is the recent observation of Gross[16,17] to the effect that saline extracts from spontaneous mouse mammary carcinomas and human cancer frequently hemolyse red cells *in vitro*. With the former preparation hemolysis was observed after 2 to 3 hours incubation whereas with the latter it became evident only after 24 hours. Weil[10] also reported a similar study in which he observed that non-necrotic tumors behaved like normal tissue while necrotic tissue apparently produced a different kind of a lytic agent. Both Weil and Gross offer their findings as the basis for an explanation of the anemias which frequently occur during malignancies.

It should be noted that in respect to time of incubation needed to produce lysis fetal liver extracts resemble extracts of spontaneous mouse mammary carcinoma.

Summary. (1) A saline extract of the liver of fetal guinea pig possesses greater hemolytic activity than a similarly prepared extract of maternal liver, as determined after 4 hours incubation at 38°C.

(2) The activity, for the most part, is due to a heat-sensitive agent and, to a lesser extent, to a heat-resistant substance.

(3) The lytic action of such extracts is not species-specific. It will lyse red cells from the same fetus, as well as those from the mother or from another species.

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Received Sept. 1, 1949. P.S.E.B.M., 1949, **72**.

Amino Acid Composition of Morphological Fractions of Rat Livers and Induced Liver Tumors.[*] (17480)

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In previous studies,[1,2] comparisons were made of the chemical compositions of the nuclear, large granule (mitochondria), small granule (microsome), and supernatant fluid fractions of the livers from normal rats, the livers of rats fed the hepatic carcinogen 4-dimethylaminoazobenzene, and the liver tumors induced by this dye. Ingestion of the dye resulted in altered levels of protein, nucleic acids, and riboflavin in certain fractions. In particular, increased levels of protein in the nuclear fraction and decreased quantities in the large and small granule fractions were observed.[1] Similar changes, but more extreme, were found in the proteins of the tumor fractions.[2] With the aid of microbiological methods developed for the determination of amino acids,[3,4] we have now determined the levels of 17 amino acids in the total proteins of the intracellular fractions from the livers of rats fed diets with or without 4-dimethylaminoazobenzene and from liver tumors. Isolation of the morphological parts of the cell permitted the detection of alterations in the composition of the cell which would have been obscured if only the whole cell proteins had been analyzed.

Experimental. Although the livers fractionated for the amino acid determinations were first perfused with saline, the degree to which contamination of the liver proteins by blood proteins might otherwise occur was determined in a preliminary study. This was calculated for each of several rats from counts of the red blood cells in the heart blood and in the perfusion fluid from the liver. The rats were killed by ether or by decapitation, and, in each case, the inferior vena cava was cannulated cephalic to the liver and ligated just caudal to the liver. The portal vein was then severed and the liver carefully excised. Isotonic NaCl was introduced through the cannula and allowed to flow out through the portal vein into a collecting vessel. The livers were weighed before and after perfusion. The livers that were fractionated were perfused in a similar manner *in situ.* For the fractionations, two groups of 4 male rats‡ weighing 180 to 200 g were fed *ad libitum,* a basal diet,[5] containing 12% of casein and 1.2 mg of riboflavin per kg. The diet for one group also contained 0.06% of 4-dimethylaminoazobenzene. After 4 weeks, the rats were killed with ether and their livers were immediately perfused. The tumor tissue was collected from the livers of 20 rats which had been fed the diet containing 0.06% of 4-dimethylaminoazobenzene for 4 to 5 months. Although only liver tumors which were less than 1 cm in diameter and which showed no gross signs of degeneration were used, histological examination showed that the tumors contained small areas of necrosis and connective tissue and numerous leucocytes.[2] The tumor-bearing livers were not perfused. These tumors were nearly white and their color was unchanged upon perfusion of the liver. This would now be expected from the recent demonstration that liver tumors are supplied only with arterial blood.[6] Furthermore, omission of this step considerably decreased the time required for the collection of the tumors. The livers or tumors were then pooled, forced through a plastic tissue mincer, homogenized in 0.88 M sucrose solution, and fractionated by differential centrifugation as described previously.[1,2,7] The perfusion, centrifugation, and all other operations prior to the precipitation of the proteins were performed at 3°C. Each fraction was suspended in sucrose solution, and aliquots from each fraction and the unfractionated homogenate were analyzed for nucleic acids.[8] The proteins in the remainder of each fraction and from an aliquot of the original homogenate were precipitated with trichloroacetic acid at a final concentration of 9% and washed once with 1 M acetate buffer (pH 5.0) and twice with 95% ethanol. Following extraction for 48 hours with ethanol at 60°C in a Soxhlet apparatus, the proteins were dried in weighing bottles *in*

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* This work was supported in part at the University of Wisconsin by grants from the National Cancer Institute, United States Public Health Service, and the Jane Coffin Childs Memorial Fund for Medical Research.

† Predoctorate Research Fellow, National Cancer Institute, 1948-49.

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TABLE I.
Distribution of Protein and Nucleic Acids in the Tissue Fractions.

Fraction	Livers from rats fed		
	Basal diet	Basal diet + dye	Rat liver tumors
	Mg protein per g fresh tissue (to the nearest whole number)		
Whole homogenate	123	106	119
Nuclei	16	16	42
Large granules	41	29	14
Small ”	18	16	12
Supernatant fluid	47	41	49
Recovery	122	102	117
	Mg of desoxypentosenucleic acid per g of fresh tissue		
Whole homogenate	1.94	2.10	5.57
Nuclei	1.89	2.01	5.00
Large granules	0.00	0.00	0.49
Small ”	0.00	0.00	0.10
Supernatant fluid	0.00	0.00	0.24
Recovery	1.89	2.01	5.83
	Mg of pentosenucleic acid per g of fresh tissue		
Whole homogenate	5.70	3.57	6.89
Nuclei	0.49	0.25	1.76
Large granules	1.80	1.06	0.76
Small ”	1.78	0.97	1.33
Supernatant fluid	1.11	0.96	2.47
Recovery	5.18	3.24	6.32

vacuo first over $CaCl_2$ and finally over H_2SO_4. This procedure was carried out quantitatively and the yields were determined by direct weighing. Analyses for ash, lipid, and glycogen indicate that approximately 97% of such preparations is a mixture of protein and nucleic acids.[9] Nitrogen was determined by the Dumas method.§

For the microbiological amino acid determinations, 40 mg of the dried preparations were autoclaved in 3 ml of 3 *N* HCl at 121°C for 16 hours, neutralized, diluted to 100 ml and appropriate aliquots were used for each assay. Previous studies have shown that the amino acids determined are essentially stable to this treatment. Leucine, valine, phenylalanine, and glutamic acid were determined with *Lactobacillus arabinosus* 17-5 as the test organism; arginine, threonine, and histidine with *Streptococcus faecalis* R as the test organism; lysine, methionine, aspartic acid, proline, serine, isoleucine, tyrosine, and cystine with *Leuconostoc mesenteroides* P-60; and glycine with *Leuconostoc citrovorum* 8081 as the test organism. All of the assays were carried out with a final volume of 2 ml per tube and with the assay conditions and media summarized in a recent publication.[10] Liver extract was also included in the medium for *Leuconostoc citrovorum* 8081.[11] Tryptophan was determined on separate 10-30 mg samples of the protein preparations by the Bates method as described by Graham *et al.*[12] Calculated on a fresh weight basis similar to that indicated in Table I for protein and nucleic acids, the sum of the amounts of each amino acid in the 4 fractions was in good agreement with the amount found in the unfractionated homogenate.

Results and Discussion. In the preliminary study, it was found that, when rats were killed by decapitation without ether anesthesia, the perfusion removed about 0.05 ml of blood per gram of liver. If the rats were killed with ether and not decapitated, approximately 0.2 ml of blood was removed per gram of liver. The livers from the rats killed with ether had the same weight before and after perfusion while the livers from decapitated rats gained approximately 15% in weight; thus in either case the saline remaining in the portal vessels accounted for about 20% of the fresh weight of the livers. Therefore, the total proteins from the unperfused livers of rats killed by decapitation or by ether anesthesia would contain approximately 6 or 22%, respectively, of blood proteins, even assuming that the blood contained as little as 20% of protein and the perfused liver as much as 15% of protein. These results are in agreement with those of Grund[13] on the perfusion of dog liver. The levels of total protein, desoxypentosenucleic acid, and pentosenucleic acid in the whole homogenates and in each of the fractions (Table I) are in agreement with previous results.[1,2] Subsequent studies have shown that increasing the casein content of the basal diet from 12 to 24% does not alter the level or distribution of protein in the livers of adult rats.[14] In order to express the amino acid data on a protein basis, the "nucleic acid-free protein" has been calculated by subtracting the quantities of the two nucleic acids from the amount of protein in the same fraction.

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§ Clark Microanalytical Laboratories, 104½ West Main Street, Urbana, Ill.

[9] Miller, E. C., and Miller, J. A., *Cancer Research*, 1947, **7**, 468.

[10] Schweigert, B. S., Guthneck, B. T., Kraybill, H. R., and Greenwood, D. A., *J. Biol. Chem.*, 1949, **180**, 1077.

[11] Sauberlich, H. E., and Baumann, C. A., *J. Biol. Chem.*, 1948, **176**, 165.

[12] Graham, C. E., Smith, E. P., Hier, S. W., and Klein, D., *J. Biol. Chem.*, 1947, **168**, 711.

The levels of arginine, histidine, lysine, leucine, valine, isoleucine, threonine, tryptophan, phenylalanine, and methionine in the nucleic acid-free proteins from the whole homogenates and from each of the fractions are presented in Table II. Similar data for proline, glycine, aspartic acid, glutamic acid, cystine, tyrosine, and serine are shown in Table III. By Dumas analysis, the protein-nucleoprotein preparation of the whole homogenate from the livers of rats fed the basal diet contained 14.5% nitrogen and the whole homogenate from the liver tumors contained 15.1% nitrogen. By calculation, the nucleic acid-free proteins from these

TABLE II. Amino Acid Composition of the Proteins of the Tissue Fractions. (Expressed as % of the nucleic acid-free proteins).

Fraction	Arginine	Histidine	Lysine	Leucine	Valine	Isoleucine	Threonine	Tryptophan	Phenylalanine	Methionine
Livers from rats fed basal diet for 4 wk										
Whole homogenate	6.44	3.20	8.84	10.66	5.76	4.92	4.26	1.49	4.97	2.91
Nuclei	7.15	2.68	9.56	10.10	5.22	4.62	4.17	1.22	3.92	2.60
Large granules	5.57	2.69	8.35	10.45	5.50	5.50	3.97	1.54	4.82	2.98
Small ,,	6.22	2.97	8.20	9.80	5.00	4.36	4.09	1.85	4.70	2.48
Supernatant fluid	5.59	2.92	8.12	10.10	5.15	4.66	3.76	1.05	4.42	2.66
Livers from rats fed basal diet + dye for 4 wk										
Whole homogenate	6.10	2.75	8.66	10.40	5.14	4.63	4.07	1.52	4.51	2.74
Nuclei	7.57	2.51	8.68	9.62	4.93	4.38	3.90	1.36	3.79	2.55
Large granules	6.71	2.55	7.94	10.65	5.64	4.77	4.15	1.42	4.48	2.71
Small ,,	6.14	2.79	7.86	9.90	5.13	4.40	3.79	2.15	4.50	2.07
Supernatant fluid	6.12	2.97	7.89	10.73	5.35	4.85	3.99	1.40	4.36	2.28
Liver tumors induced by feeding dye 4 to 5 mo.										
Whole homogenate	6.32	2.75	8.45	9.45	4.80	4.53	3.92	1.39	3.74	1.95
Nuclei	6.98	2.26	8.06	8.52	4.59	3.91	3.64	1.09	3.30	1.54
Large granules	5.90	2.20	7.94	9.80	5.15	4.53	3.27	1.68	3.90	2.14
Small ,,	5.38	2.40	7.53	9.15	4.80	4.25	3.64	1.62	3.87	1.89
Supernatant fluid	5.83	2.71	8.15	9.58	5.25	4.30	4.30	1.03	3.89	2.00

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[13] Grund, G., *Z. Biol.*, 1910, **54**, 173.

[14] Price, J. M., Miller, E. C., Miller, J. A., and Weber, G. M., *Cancer Research*, in press.

TABLE III.
Amino Acid Composition of the Proteins of the Tissue Fractions
(Expressed as % of the nucleic acid-free proteins)

Fraction	Proline	Glycine	Aspartic acid	Glutamic acid	Cystine	Tyrosine	Serine
			Livers from rats fed basal diet 4 wk				
Whole homogenate	4.00	4.01	8.38	9.87	.58	3.99	4.05
Nuclei	4.37	5.62	8.06	8.85	.46	3.71	3.89
Large granules	3.90	4.16	8.17	9.20	.52	3.87	4.08
Small ”	3.78	3.37	7.79	9.20	.55	4.09	3.98
Supernatant fluid	4.20	3.71	7.99	10.28	.71	3.70	3.76
			Livers from rats fed basal diet + dye 4 wk				
Whole homogenate	4.21	3.97	7.61	9.81	.64	3.93	3.95
Nuclei	4.24	5.83	6.87	11.35	.46	3.45	4.19
Large granules	4.05	3.90	7.13	10.44	.49	3.86	3.94
Small ”	3.64	3.45	7.54	9.20	.59	4.19	4.08
Supernatant fluid	3.81	3.74	8.20	11.10	.85	3.84	3.87
			Liver tumors induced by feeding dye 4 to 5 mo.				
Whole homogenate	4.07	4.86	7.72	11.11	.74	3.66	4.30
Nuclei	4.24	6.93	7.26	10.83	.60	3.63	4.28
Large granules	3.61	4.10	7.79	9.68	.75	4.02	4.29
Small ”	3.41	3.56	7.80	9.79	.63	3.68	4.34
Supernatant fluid	3.66	3.58	8.35	11.41	.89	3.99	5.00

samples contained 14.5 and 14.9% of nitrogen, respectively. Through an oversight, none of the protein sample from the whole homogenate of the livers of the rats fed the dye was reserved for nitrogen analysis. The 17 amino acids determined accounted for 85 and 79% of the nitrogen in the nucleic acid-free proteins from the whole homogenates of the livers of the rats fed the basal diet and of the liver tumors. Analyses for alanine with *Leuconostoc citrovorum* indicated that the liver proteins from the rats fed the basal or dye-containing diets and the tumors contained approximately 6% of alanine. The inclusion of alanine increased the nitrogen recoveries to 91 and 86%, respectively, for the nucleic acid-free proteins from the livers of the rats fed the basal diet and the liver tumors.

Table IV lists those amino acids whose concentrations in the nucleic acid-free proteins from the various fractions differed from their concentrations in the proteins from the unfractionated tissues by 15% or more. In general, the proteins from the nuclear fractions contained less histidine, tryptophan, cystine, and phenylalanine and about 40% more glycine than the proteins from the unfractionated tissues. The proteins from the large granules usually had a relatively low concentration of histidine, while those from the small granules generally had a lower concentration of methionine, proline, and glycine and a higher concentration of tryptophan than the whole homogenate proteins.

In each case the supernatant fluid proteins contained more cystine than whole liver protein. Melampy[15] has shown that the whole nuclei of chicken erythrocytes contain less histidine, tryptophan, and phenylalanine than the cytoplasm; no analyses for cystine or glycine were reported. The livers of the rats which had been fed the dye for 4 weeks contained 20 to 33% higher concentrations of glutamic acid in the nuclear proteins, arginine in the large granule proteins, and cystine and tryptophan in the supernatant fluid proteins than were found in the corresponding fractions from the normal livers, Table V. The tumor proteins differed from the normal liver proteins even more than did those from the livers of the rats fed the dye. The methionine contents of the proteins from each fraction were decreased by 25 to 41% while the cystine contents were increased by 15 to 44%. However, the levels of cystine in these proteins

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[15] Melampy, R. M., *J. Biol. Chem.*, 1948, **175**, 589.

TABLE IV.
Summary of Differences Between Amino Acid Composition of Proteins from Fractions and the Whole Homogenates Which Exceeded 15%.
(Expressed as % higher (+) or lower (—) in concentration in the fraction).

	Livers from rats fed					
Fraction	Basal diet		Basal diet + dye		Rat liver tumors	
Nuclei			Arginine	+24		
	Histidine	—16			Histidine	—18
	Tryptophan	—18			Tryptophan	—22
	Phenylalanine	—21	Phenylalanine	—16		
					Methionine	—21
	Glycine	+40	Glycine	+47	Glycine	+43
			Glutamic acid	+17		
	Cystine	—21	Cystine	—28	Cystine	—19
Large granules	Histidine	—16			Histidine	—20
					Threonine	—17
					Tryptophan	+21
					Glycine	—16
			Cystine	—23		
Small granules					Arginine	—15
	Tryptophan	+24	Tryptophan	+41	Tryptophan	+17
	Methionine	—15	Methionine	—24		
			Proline	—16	Proline	—16
	Glycine	—16			Glycine	—27
					Cystine	—15
Supernatant fluid	Tryptophan	—30			Tryptophan	—26
			Methionine	—17		
					Glycine	—26
	Cystine	+22	Cystine	+33	Cystine	+20
					Serine	+16

were relatively low and the cystine assay is less reliable than the other microbiological assays used. The concentrations of glycine and glutamic acid in the nuclear proteins and of serine in the supernatant fluid proteins were also increased by 23 to 33%, respectively. Whether the alterations found following the ingestion of the carcinogen were due to changes in the relative amounts of the various proteins or to alterations in the composition of specific proteins will require much further study.

Summary. 1. The perfused livers from adult rats fed either a basal diet containing 12% of casein or the same diet plus 4-dimethylaminoazobenzene for 4 weeks, and the liver tumors induced by this dye were fractionated into nuclear, large granule (mitochondria), small granule (microsome), and supernatant fluid fractions. The amounts of 17 amino acids were determined in the total proteins from the original homogenate and in each of the fractions. The importance of using perfused livers for these analyses was demonstrated experimentally.

2. When the basal diet was fed, the proteins from the nuclear fraction contained lower concentrations of histidine, tryptophan, phenylalanine, and cystine and a higher concentration of glycine than the whole liver proteins. The large granule proteins contained less histidine, the small granule proteins less methionine and glycine and more tryptophan, and the supernatant fluid proteins less tryptophan and more cystine than the proteins of the original homogenate.

3. The livers of rats fed the dye contained higher concentrations of glutamic acid in the nuclear proteins, arginine in the large granule proteins, and cystine and tryptophan in the supernatant fluid proteins than normal livers.

4. The proteins from each fraction of liver tumor contained less methionine and more cystine than the proteins in the corresponding

TABLE V.
Summary of Amino Acids Which Occurred in Livers of Rats Fed Dye and in Tumors at Levels That Differed from Normal Liver by 15% or More.
(Expressed as % higher (+) or lower (—) in concentration than in normal liver).

Fraction	Livers from rats fed basal diet + dye		Rat liver tumors			
Whole homogenate			Methionine	—33		
			Cystine	+28		
			Glycine	+21		
			Phenylalanine	—25		
			Valine	—17		
Nuclei	Aspartic acid	—15				
			Methionine	—41	Histidine	—16
			Cystine	+30	Isoleucine	—15
			Glycine	+23	Lysine	—16
			Phenylalanine	—16	Leucine	—16
	Glutamic acid	+28	Glutamic acid	+22		
Large granules	Arginine	+20				
			Methionine	—28	Histidine	—18
			Cystine	+44	Isoleucine	—18
			Phenylalanine	—19	Threonine	—18
Small granules	Tryptophan	+16				
	Methionine	—17	Methionine	—24	Histidine	—19
			Cystine	+15		
			Phenylalanine	—18		
Supernatant fluid	Tryptophan	+33				
			Methionine	—25		
	Cystine	+20	Cystine	+25		
			Serine	+33		

fractions of normal liver. In addition, the concentrations of glutamic acid and glycine in the nuclear proteins and of serine in the supernatant fluid proteins were higher in the tumor tissue than in normal liver.

Received October 20, 1949. P.S.E.B.M., 1949, **72**.

Utilization of Guanine by the C57 Black Mouse Bearing Adenocarcinoma Eo771.[*] (17481)

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The effectiveness of the triazole analogue of guanine, azaguanine, in inhibiting the growth of subcutaneous transplants of mammary adenocarcinoma Eo771[1] in mice of the C57 black strain has been reported by Kidder, Dewey, Parks and Woodside[2] and confirmed for this tumor.[3] It was suggested by Kidder and co-workers[2] that their results indicated that the tumor tissue utilizes guanine for nucleic acid synthesis while normal tissue does not.

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[*] The authors wish to acknowledge the support of the National Cancer Institute, United States Public Health Service, the Office of Naval Research, and the James Foundation of New York, Inc.

[1] Snell, G. D., Cloudman, A. M., and Woodworth, E., *Cancer Res.*, 1948, **8**, 429.

[2] Kidder, G. W., Dewey, V. C., Parks, R. E., Jr., and Woodside, G. L., *Science*, 1949, **109**, 511.

We have investigated the incorporation of isotopically labeled guanine (containing N^{15} in the 1 and 3 nitrogens and in the 2-amino group[4]) into the nucleic acids of this strain of mouse bearing this tumor. The guanine was administered in five intraperitoneal injections, of 2.4 mg each, over a 6 day period, beginning on the 7th day after implantation of the tumor. Inasmuch as isotopic nitrogen, particularly from the 2-amino group, can be contributed to body pool ammonia[4] it is not unlikely that the isotope found in the protein residue, pyrimidines and even the adenine (Table I) arose from this source. There was, however, a small but definite incorporation into the guanine of the nucleic acids, but there is no evidence of a specific uptake by the tumor since the incorporation into the guanine of the tumor tissue and into that of the non-tumorous viscera of the same mice was significant in each case.

Incorporation of Guanine into Nucleic Acid of C57 Mice Implanted with Adenocarcinoma Eo771.

	Atom % excess N^{15}
Injected guanine	6.40
Viscera:	
Nucleic acids	.030
Total purines	.049
Guanine	.072
Pyrimidines	.024
Tissue residue (after extraction of nucleic acids)	.015
Tumor:	
DNA	
Total purines*	.046
Adenine†	.038
Guanine	.050
PNA	
Total purines*	.058
Adenine†	.037
Guanine	.105

* Isolated as the copper salts after separation of the pentose (PNA) and desoxypentose (DNA) nucleic acids by the Schmidt-Thannhauser procedure.

† Calculated from N^{15} values of adenine picrate.

When guanine was adminisered to an unspecified strain of the white rat[5] or to Sherman strain rats (Rockland Farms), either orally[4] or intraperitoneally,[6] there was no significant incorporation of the guanine into the tissue nucleic acids. The species difference in the purine metabolism of the Sherman rat and the C57 black mouse (bearing adenocarcinoma Eo771) makes it necessary to be conscious of other such possibilities in mammalian purine metabolism.

Received October 17, 1949. P.S.E.B.M., 1949, **72**.

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[3] Sugiura, K., Hitchings, G. H., Cavalieri, L. F., and Stock, C. C., in press.

[4] Brown, G. B., Roll, P. M., Plentl, A. A., and Cavalieri, L. F., *J. Biol. Chem.*, 1948, **172**, 469.

[5] Plentl, A. A., and Schoenheimer, R., *J. Biol. Chem.*, 1944, **153**, 203.

[6] Brandt, A., Roll, P. M., and Brown, G. B., unpublished data.

Behavior of Thyroid Toward Elements of the Seventh Periodic Group. II. Rhenium.[*][†] (17482)

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On the basis of speculations made in a previous report[1] in which we suggested that not only iodine but all the elements of the seventh periodic group are selectively filtered by the thyroid, we have extended our work to include rhenium. As with Cl, Br, I, Mn and At, we find that Re also accumulates in the thyroid in greater concentration than in any other tissue examined, thus lending further support to our hypothesis.

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[*] A report of this work was made before the American Society of Biological Chemists at Detroit, May 1948. *Fed. Proc.*, 1949, **8**, 182.

[†] Aided in part by grants from the American Cancer Society and from the Division of Grants and Fellowships of the National Institutes of Health, United States Public Health Service.

[1] Baumann, Emil J., and Metzger, N., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 536.

Metallic Re powder was irradiated in the Oak Ridge nuclear reactor. It was dissolved in 2 ml of concentrated nitric acid and appropriately diluted for parenteral administration. This perrhenic acid solution contained 2 radioactive Re isotopes, Re^{186} and Re^{188} having half lives of 90 and 18 hours, respectively, as well as a large amount of stable Re. In nearly all the experiments to be described, the 18 hour half life isotope was allowed to decay to such an extent that by the time of our experiments it was an unimportant factor in our measurements. Of necessity, however, a relatively large amount of carrier Re was given with each dose—from 3 to more than 100 μg depending on the age of the irradiated Re. The amount of stable Re given in each case was therefore greater than a tracer quantity.

Adult albino rats weighing 250 to 400 g were fed on a diet consisting of mixed cereals, dried milk, meat scrap, yeast, and salts moistened with fresh milk. Fresh vegetables were given separately. Since we were studying only the filtration process of the thyroid, it was desirable to inhibit completely the reactions whereby thyroxine and diiodotyrosine are synthesized. To this end, thiouracil[‡] was added to the mixture to make a 1% concentration. For comparison with normals, however, thiouracil was omitted from the food of 8 rats.

One ml of a HRe^*O_4 solution having an activity of 200,000 to 300,000 counts per minute[§] was injected intraperitoneally into each rat. The animals were sacrificed with $CHCl_3$ usually 4 hours after the injection. In a number of instances in which the effect of time on the Re* concentration of tissues was studied, rats were killed 1 to 24 hours after injection. Autopsies were performed at once and tissues prepared for analysis. As much blood as possible was drained from the tissue before hashing. One to 2 g samples of blood, lung, liver and muscle were weighed into 15 mm diameter metal planchets, moistened with 10-20 drops of 1% NaOH, dried for an hour at 105°C and then at 120-130°C. The thyroid of each rat was weighed in a planchet, moistened with 1-2 drops of NaOH and dried in the same way. The samples were then ashed at 450-500°C for 18 hours. The ash was coated with 0.5-1.0 ml of 1% celloidin and the activity measured with a thin mica window Geiger counter. Appropriately diluted samples of the injected solution measured with the same apparatus were used as standards to establish the decay curve for the Re* preparation in each series of experiments. Known amounts of Re* were added to blood and to muscle samples and ashed as described. The maximum loss in these controls due to self absorption and possibly to some loss during ashing was found to be not more than 15% and usually less than 10%. No corrections are made for these errors in the data given.

Fig. 1 shows the distribution of Re* in thyroid, lung, liver, muscle, and blood of 8 normal rats measured as described. Fig. 2 shows similar observations on 8 representative rats which had been under the influence of thiouracil for from 5 to 20 days before Re* was injected. The thyroids weighed 45 to over 100 mg depending on the duration of thiouracil feeding. They were very hyperemic and hyperplastic. The thyroids of the control rats weighed 14 to 20 mg, a few being slightly enlarged and slightly hyperemic.

Four hours after Re* injection, an average of 4.5% of the administered dose per g of tissue was found in the thyroids of normal rats, the range was 1.4 to 7.2% per g. For thiouracil treated rats which had been given 1 ml of a Re* solution containing 3 to 12 μg of carrier, the average concentration was 12.8% per g of thyroid with a range of 5.1 to 25.5% per g. When larger amounts of carrier were used, smaller proportions of the injected dose were found in the thyroids. For example, with 4 thiouracil treated rats a Re* solution containing 250 μg Re carrier

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[‡] We thank the American Cyanamid Company for supplying the thiouracil.

[§] The activity of the Re shipments varied from 25,000 to 100,000 cpm per μg at the time of shipment as determined from data submitted with the irradiated preparations and from the catalogue of isotopes of the Atomic Energy Commission.

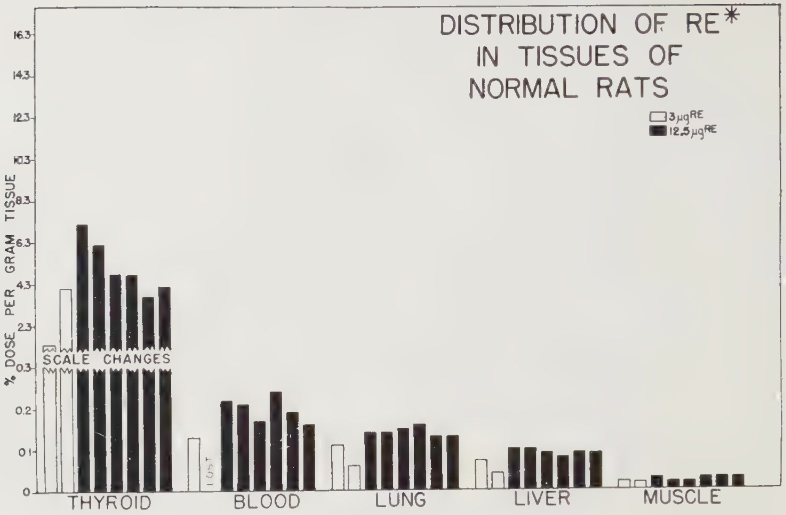

FIG. 1.

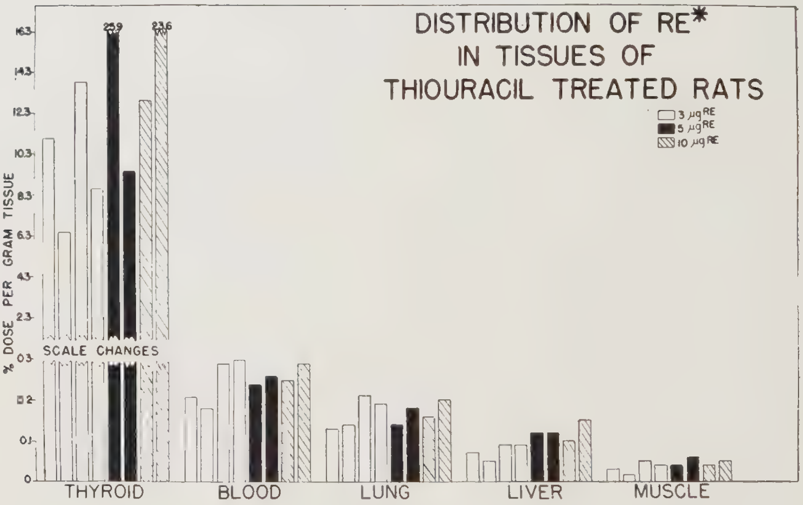

FIG. 2.

per ml was injected; 4 hours later, the thyroids were found to contain only 2.2 to 4.2% of the dose per g of tissue. Since the capacity of any tissue to take up any substance is limited, it is to be expected that with increasingly large amounts, a smaller fraction of the administered dose will be retained. As with iodine, the percentage of administered Re* that can be taken up varies in a general way, directly with the mass of thyroid and inversely with amount of carrier.

The amount of Re* found in other tissues was very much smaller—only 0.5% to 5% of the injected Re* per g of tissue. The Re* concentrations of blood, liver, lung and muscle of the thiouracil treated rats differed little from the corresponding concentrations found in the control group. On the average, lung of normal rats contained 0.13% of the injected Re* per g and in thiouracil fed rats 0.16%. Corresponding average figures for liver were 0.08% and 0.10%, and for muscle 0.03% and 0.04%, respectively. In all, experiments were made on 41 thiouracil fed rats with similar results in every case. Fig. 3 shows a comparison of the average Re* uptake of the 8 normal and the 8 representative thiouracil fed rats shown in Fig. 1 and 2.

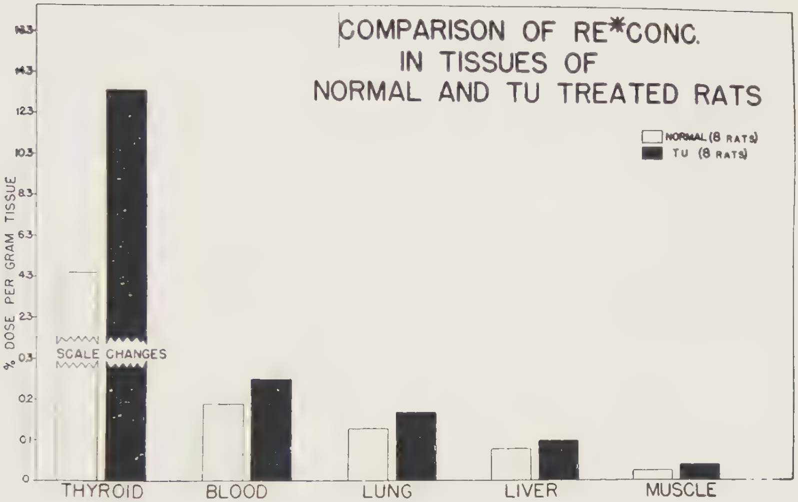

FIG. 3.

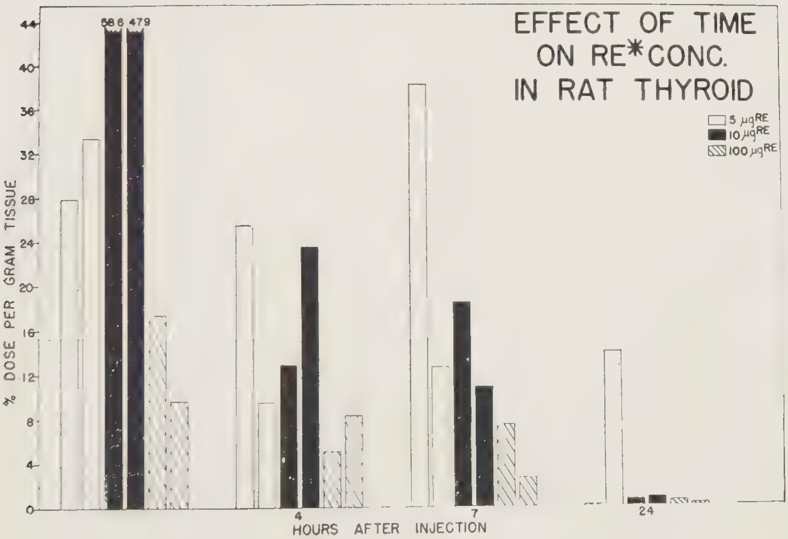

FIG. 4.

The ratio of the Re* concentration in the thyroid to that in the blood is invariably large, 25 to 100 or more; while the ratio of Re* concentration of other tissues to blood is always less than one. It follows, therefore, that the process whereby such a high thyroid Re* content comes about is an active selective filtration, and not simply a passive reflection of the amount of Re* in the blood passing through this organ.

In view of the common origin of both thyroid and lung from the primordial gill clefts, it is worthy of note that the Re* concentration of lung is exceeded only by that of the thyroid.

To determine the effect of time on the distribution of the Re* in the body after injection of a single dose, 3 experiments were performed. In each case 1 ml of a Re* solution was injected intraperitoneally into 8 thiouracil fed rats. At 1, 4, 7 and 24 hours after injection 2 animals were sacrificed. The amount of carrier given per rat in these 3 experiments was 5, 10 and 100 μg and the activity of the preparations per ml was 270,000, 282,000, and 431,000 c/m, respec-

TABLE I.
Excretion of Injected Re* (%).

	Re injected		Urine			Feces		
	Carrier, mg	Re* activity, cpm, ×1000	1st day	2nd day	Total	1st day	2nd day	Total
Rat 12a	0.010	309	84.4	0.7	85.1			
'' 12b	0.010	309	79.4	2.2	81.6			
Rabbit 14b	0.50	1310	94.8	1.1	95.9	.1	.8	.9
'' 16a	1.00	492	79.8	12.4	92.2	.0	.0	.0
'' 16b	1.00	492	92.1	2.8	94.9	.0	.0	.0

tively. The uptake of Re* by the thyroids is shown in Fig. 4.

In all tissues examined, the greatest concentration of Re* usually appeared at the one hour interval, decreasing rather slowly to 7 hours after injection, and then more rapidly; so that 24 hours after, not more than ⅓ and usually less than 1/10 of the amount found one hour after the injection remained. After another day, the tissue activity was little more than at background levels. Although there was considerable variation among individuals (*e.g.* see Fig. 4 at the 24 hour interval) this general statement of our findings held whether 5, 10, or 100 μg of carrier were given. Again, with the larger doses as, *eg.*, when 100 μg of carrier Re were given, the percentage of the dose found in the thyroid and other tissues was much less than with the smaller amounts of carrier.

From these data one may assume that Re is rapidly excreted. From the urines of 2 rats, 85 and 82% of an intraperitoneal Re injection was recovered in 2 days of which all except 1-2% was excreted in the first day. Because of the difficulty of collecting quantitatively the little urine excreted by a rat, similar experiments were made with 3 rabbits. Bladders were compressed at the end of each day. Here 96, 92, and 95% of the injected Re was recovered in 2 days. The feces of 2 rabbits showed negligible radioactivity, while 0.9% of the injected dose was recovered in the feces of the 3rd rabbit. Contamination of feces by urine could not be excluded. The details of these tests are given in Table I.

Total recovery of injected Re* was attempted on 2 rats 3.5 hours after intraperitoneal injection of 1 ml of a Re* solution containing 13 μg of carrier Re, having an activity of 125,000 c/m. The 2 rats were sacrificed with $CHCl_3$ and tissues prepared for analysis. Only 72% and 85%, respectively, were recovered. The Re* was distributed as follows:

	Rat 1, %	Rat 2, %
Thyroid	1.09	1.23
Liver	1.32	1.24
Skeleton and muscle	12.6	14.8
Blood	2.33	1.63
Other viscera	25.4	16.7
Skin and hair	15.1	25.2

A large part of the unrecovered rhenium can be accounted for by the loss of urine during anesthetization. In general, the distribution is similar to that known for chlorine.

Summary. Rhenium is preferentially and actively filtered from the blood by the thyroid. After injecting a single dose, the Re* concentration in that organ is 25 to 100 or more times greater than that found in any other tissue. These findings therefore add support to our hypothesis that the thyroid will selectively filter from the blood all the elements of the seventh periodic group. The greatest concentration of Re* in tissues occurs within one hour. For several hours it decreases slowly and then more rapidly so that more than 90% is recovered from the urine in 24 hours.

Received October 18, 1949. P.S.E.B.M., 1949, **72**.

Immunologic Response of Hamsters to Influenza Virus Strains.* (17483)

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(Introduced by W. Henle)

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Studies on strains of influenza virus isolated from the 1947 epidemic[1] were complicated by irregular reactions obtained in hemagglutination-inhibition tests with chicken sera (*e.g.*, nonspecific inhibition). In view of these difficulties, it was deemed desirable to supplement the antigenic studies with complement-fixation tests. Since chicken sera are unsatisfactory for this purpose, antiserum had to be prepared in another species. The hamster was selected because of its ability to develop antibodies to influenza virus following inoculation by the intranasal route.[2] The results obtained with hamster sera are the subject matter of this paper.

Methods and Materials. Viruses. The following virus strains were used:

PR8 and Lee
$L_3$47, $L_7$47, and $L_8$47
FM_1[3]
$L_4$49 and $L_8$49[4]
FJS (type A) and Warner (type B)†

Antigens. Allantoic fluids harvested 48 hours after intraallantoic inoculation of 10^{-3} or 10^{-4} dilution of seed virus which were centrifuged at 2,000 rpm for 10 minutes and dialyzed overnight against 20 parts of phosphate buffer saline were used as the "virus-bound" antigen. Obviously these preparations contained some soluble antigen. No attempt was made to separate the two antigens from the fluids. Ten or 20% suspensions of allantoic membrane were centrifuged at 2,000 rpm for 10 minutes, and following removal of the virus (as determined by absence of hemagglutination) by either centrifugation at 13,000 rpm for one hour or absorption with red cells, or a combination of both procedures, the supernatants were used as the soluble antigen. Suspensions of uninfected membranes were used as control antigens.

Sera. Hamsters were inoculated by the intranasal route with 0.1-0.2 cc of active infected allantoic fluids containing virus. Fifteen days later they were bled either with needle and syringe from the heart or by incision of the thoracic cavity and heart, allowing blood to run through funnel into tube. The sera were inactivated by heating at 56°C for 30 minutes. Before being used in the hemagglutination-inhibition test, they were absorbed with human or chicken erythrocytes, depending on which red cells were to be used in the test.

Hemagglutination-inhibition Test. The procedure for performing the hemagglutination-inhibition test has been described previously.[5] The tests were read after 30-45 minutes of incubation at room temperature. In the presence of hamster sera—especially in the lower dilutions—there was a tendency on the part of the cells to settle out quickly, so that the tubes with agglutinated cells showed patterns consisting of fuzzy, serrated rings, or clumps of cells against a background of a more or less smooth film of cells (the kind of film usually observed on the bottom of tubes showing agglutination of cells in the presence of human or chicken sera).

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* This research was conducted under contract with the Office of Naval Research.

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TABLE I.
Cross-hemagglutination-inhibition Reactions with Hamster Antisera.

	Serum						
Antigen	PR8	FJS	FM$_1$	L$_3$47	L$_7$47	L$_8$47	Lee
PR8	*2560*	20	20	<15	<15	15	<15
FJS	<15	*320*	20	15	15	<15	<15
FM$_1$	<15	60	*320*	240	80	80	<15
L$_3$47	<15	40	240	*320*	120	60	<15
L$_7$47	<15	<15	60	60	*120*	30	<15
L$_8$47	<15	15	30	30	40	*240*	<15
Lee	<15	<15	<15	<15	<15	<15	*80*

TABLE II.
Cross-complement-fixation Test with Allantoic Fluid Antigens and Hamster Sera.

		Serum			
Antigen	Dilution	PR8	L$_7$47	L$_4$49	L$_8$49
PR8	und.	192*	<12	<12	<12
	1:2	192	<12	<12	<12
	1:4	96	<12	<12	<12
	1:8	192	<12	<12	<12
	1:16	24	<12	<12	<12
	1:32	<12	<12	<12	<12
L$_7$47	und.	12	48	24	24
	1:2	12	96	48	24
	1:4	24	96	48	48
	1:8	24	96	48	48
	1:16	12	48	48	24
	1:32	<12	24	<12	<12
L$_4$49	und.	12	48	48	48
	1:2	24	24	48	24
	1:4	24	48	48	48
	1:8	12	48	48	48
	1:16	12	24	24	48
	1:32	<12	12	<12	<12
L$_8$49	und.	12	nt	24	48
	1:2	24	nt	48	48
	1:4	24	nt	48	96
	1:8	12	nt	48	48
	1:16	12	nt	48	24
	1:32	<12	nt	<12	<12

* Serum titer expressed as the reciprocal of serum dilution.

Complement Fixation Test. The procedure of the complement fixation test was as follows: Serial, two-fold dilutions of serum in 0.1 cc amounts were mixed with 0.1 cc amounts of progressively diluted antigen and with 0.2 cc of complement diluted to contain 1.5-2.0 units. The tubes were kept overnight in the refrigerator. The binding of complement was determined by the addition of 0.2 cc of sensitized cells (consisting of 0.1 cc of 2% sheep cells plus 0.1 cc of optimally diluted hemolysin) and incubation at 37°C for 30 minutes. The titers represent initial dilution of serum, and are based on 3+ or 4+ readings.

Results. As shown in Table I, hamster sera gave highly specific reactions in the hemagglutination-inhibition test. Not only was there no reaction between the strains from 1947-1948 and the anti-Lee serum, but there was very little or no cross-relationship between these strains and the PR8 virus. Moreover, the recent strains exhibited antigenic differences among themselves. It appears that the L$_3$47 and FM$_1$ viruses are probably identical strains.

TABLE III.
Cross-complement-fixation Test with Soluble Antigens and Hamster Sera.

Antigen	Dilution	Serum			
		PR8	$L_7$47	$L_4$49	$L_8$49
PR8	1:2	48	12	24	12
	1:4	48	12	24	12
	1:8	24	12	24	24
	1:16	48	12	12	24
	1:32	48	<12	<12	<12
	1:64	12	<12	<12	<12
$L_7$47	1:2	12	24	48	24
	1:4	12	24	24	24
	1:8	12	24	24	24
	1:16	<12	12	<12	<12
	1:32	<12	<12	<12	<12
	1:64	<12	<12	<12	<12
$L_4$49	1:2	12	12	24	24
	1:4	12	12	24	24
	1:8	12	12	24	24
	1:16	<12	12	12	24
	1:32	<12	<12	<12	<12
	1:64	<12	<12	<12	<12
$L_8$49	1:2	12	nt	24	24
	1:4	12	nt	24	24
	1:8	12	nt	24	24
	1:16	<12	nt	12	12
	1:32	<12	nt	<12	<12

Using the complement-fixing allantoic fluid antigen of influenza virus, the 1947 and 1949 strains were distinctly set off from the PR8 virus by the lack of reaction—in the dilutions used—between their sera and the antigen of the PR8 virus (Table II). The anti-PR8 serum with a higher homologous titer than the other antisera reacted with the recent strains. Although when tested with the PR8 antigen in suboptimal dilution (1:16) the homologous and heterologous titers of this serum were the same, with higher concentrations of the PR8 antigen the homologous titer was 4 to 16 times greater than the heterologous titers.

As shown in Table III, the complement-fixation test with the soluble antigens brought out the kinship of these strains. The results with the PR8 antiserum resembled the corresponding findings with the "allantoic fluid" antigen, but the results with the other sera differed from those obtained with the allantoic fluid antigen in that these sera reacted with the PR8 soluble antigen to a titer of about the same magnitude as the homologous titers. This reaction was type-specific. There was no reaction between any of these sera shown in Tables II and III and the Lee virus antigens.

Discussion. The fact that hamster antisera are active in complement fixation as well as hemagglutination tests should make them very useful in the typing of newly isolated strains.

Taylor[6] has employed hamster sera in antigenic analyses of strains of influenza virus. In our laboratory it was possible to type the viruses isolated from the outbreak of influenza of 1949[4] by such a procedure on primary isolation. The membranes of embryos used for primary isolation served as source of the soluble antigen. Once the type of virus has been established, strain differentiation can be accomplished by agglutination-inhibition tests using hamster sera. A general idea about the relationship of the isolated virus to other newly identified strains (subtype specificity) may be obtained from the complement-fixation reaction with allantoic fluid containing virus antigen. Another advantage in using hamsters is the ability of these animals to produce antibodies in response to low concentrations of virus. This makes it possible to prepare antisera to viruses in first or second egg passage. This is not always feasible with chickens because more virus is required to induce antibody formation in these animals. The value of hamster sera is slightly affected by a shortcoming; the hemagglutination-inhibition test is difficult to read because of the rapid settling of cells, giving rise to fuzzy or serrated discs of agglutinated cells.

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Summary. Antisera prepared in hamsters by the intranasal instillation of allantoic fluids containing influenza virus were type-specific when used in the complement-fixation test with the soluble antigens; group- or subtype-specific (*e.g.* A prime) when used in the complement fixation test with the allantoic fluid antigen, and strain-specific in hemagglutination-inhibition tests.

It appears that the hamster is an animal best suited for quick identification of influenza viruses. It is suggested that their sera be used for systematic classification of strains.

Received October 5, 1949. P.S.E.B.M., 1949, **72**.

Effect of Epinephrine, Antihistaminics and Adrenergic Blockade on Egg White Edema in Adrenal Insufficient Rats.* (17484)

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Recently we reported a marked protection by 0.1 mg/kg epinephrine hydrochloride against the acute edema induced by the intraperitoneal injection of egg white in rats.[1] Selye[2] and Leger and Masson[3] showed that adrenalectomy greatly sensitized rats to this edema and led to death in a large percentage of the animals. Adrenal cortical extract protected such animals from dying *but not from the edema,* nor did it confer protection to animals with intact adrenals. Kellaway,[4] Wyman,[5] and Perla and Marmorston-Gottesman,[6] Wyman[7] and Ingle, *et al.*[8,9] showed that epinephrine antagonized histamine toxicity in adrenalectomized rats. The egg white edema syndrome could conceivably be caused by the local release of histamine or a histamine-like substance, since antihistaminics effectively combat the edema, as demonstrated by Leger and Masson,[10] Brown and Werner,[11] and ourselves.[1] The doses of antihistaminics required are considerable, however, which may also suggest that the protection results from precapillary arteriolar vasoconstriction[12] and decreased capillary filtration rather than "anti-

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* Supported by a grant from the Life Insurance Medical Research Fund.

† Published with the approval of the Chief Medical Director, Veterans Administration. The statements and conclusions published by the first author are a result of his own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

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TABLE I. Exp. 1.

Group	% incidence of edema	Degree of edema	% dead	Symptoms
1. Controls	100	3.5+	0	—
2. Adx untreated	80	3.5	10	30% cyanotic and all prostrated
3. Adx + epinephrine	0	0	0	None
4. Adrenal demedullated	80	4.0	0	”

TABLE II. Exp. 2.

Group	% incidence of edema	Degree of edema	% dead	Symptoms
1. Untreated controls	80	4	0	—
2. Adx untreated	100	4	100	Died in 1½-3 hr
3. Adx + epinephrine	50	3	0	Good condition
4. Adrenal demedullated (untreated)	75	4	0	—

histaminic action." Selye[2] and Leger and Masson[3] found that among other things, "alarming stimuli" or stress such as exercise, cold, India ink and formalin injections inhibited the reaction. Since epinephrine protects against egg white edema in the normal rat and against the enhanced histamine sensitivity in the adrenalectomized rat, it seemed possible that it would protect against the increased egg white sensitivity and mortality in adrenalectomized rats as effectively or more so than adrenal cortical extract. Since it was considered possible that "alarming stimuli" may act by releasing endogenous epinephrine, additional experiments were done in adrenal demedullated rats and rats in which adrenergic blockade was induced.

Methods and Results. The methods of producing and grading the severity of the edematous symptoms have been described elsewhere.[1] Two to 4 hours after the intraperitoneal injection of fresh, filtered (or redissolved lyophilized) egg white, the edema of the face and extremities was graded by the per cent incidence and by the 0–4+ degree of edema. In addition, where adrenal insufficiency was included, the per cent mortality and symptoms were recorded. Leger and Masson's observations of enhanced sensitivity following adrenalectomy were confirmed. Following adrenalectomy, the rats were kept on the stock diet (Purina Chow) and 1% NaCl + 0.2% $NaHCO_3$ drinking fluid for some time before they were given egg white. If the salt therapy was continued up to and following the injection, there were fewer deaths than if they were deprived of salt for 24 hours, but in any case the symptoms were much more severe than in animals with intact adrenals.

Exp. 1. Four groups of 10 male rats (Slonaker strain) each, average body weight 270 g were given egg white, 0.5 cc per rat, intraperitoneally. The operations were performed 10 days prior to injections, and the adrenalectomized rats were kept on salt throughout the experiment. The epinephrine-HCl (Parke, Davis natural product), 0.1 mg/kg was injected subcutaneously at the same time as the egg white. The symptoms were graded 4 hours afterward.

Adrenalectomy enhances the toxicity of the egg white and the severity of the symptoms. Epinephrine affords complete protection, in contrast to adrenal cortical extract.[3] Adrenal demedullation alone does not aggravate the symptoms.

Exp. 2. Four groups of 4-5 males each, average body weight 250 g were injected with 1.0 cc egg white per rat. The symptoms were graded 3 hours later. The operations were performed 19 days before the injections, and the adrenalectomized rats were kept on salt until 24 hours before the injections, at which time water was substituted. The epinephrine-HCl, 40 micrograms/kg was injected sub-

TABLE III. Exp. 3.

Group	% incidence of edema	Degree of edema	% dead	Symptoms
1. Untreated controls	100	4.0	0	—
2. Adx (on salt throughout)	100	4.0	80	Cyanotic and prostrated
3. Adx + epinephrine (on salt throughout)	100	4.0	25	< group 2
4. Adx (off salt 1 day)	80	3.5	80	Cyanotic and prostrated
5. Adx + epinephrine (off salt 1 day)	80	2.5	40	< group 3

TABLE IV. Exp. 4.

Group	% incidence of edema	Degree of edema	% dead	Symptoms
1. Untreated controls	100	4.0+	0	—
2. " " + SY-28	0		0	None
3. Adx untreated	100	4.0	80	Cyanotic and prostrated
4. Adx untreated + SY-28	0		0	Prostrated

cutaneously at the same time as the egg white (which is a sub-effective edema-preventative dose.)[1]

Doses of epinephrine which fail to prevent egg white edema even in animals with intact adrenals, completely protect against the fatal effects of egg white intoxication which kills 100% of the untreated adrenalectomized rats. Adrenal demedullation alone does not enhance the edema.

Exp. 3. Five groups of 5 males each, average body weight 180 g, were given egg white 0.5 cc per rat. The symptoms were graded 2 hours later. The adrenalectomies were performed 14 days before the injections, and the animals were maintained on salt both throughout the experiment, and withheld 24 hours prior. The epinephrine-HCl, 0.2 mg/kg was injected subcutaneously at the same time as the egg white injections.

As in Exp. 2 vs 1, intensification of adrenal insufficiency by salt deprivation enhances the susceptibility of adrenalectomized rats, but epinephrine still exerts its protection, in confirmation of the previous two experiments.

Exp. 4. Four groups of 5 males each, average body weight 180 g were given egg white 0.5 cc per rat. The symptoms were graded 2 hours later. The adrenalectomies were performed 14 days before the injections, and the rats were maintained on salt throughout the experiment. "SY-28", a β-haloalkyl amine (dibenamine congener) adrenergic blocking drug, N-(2-bromoethyl)-1-naphthalene-methylamine hydrobromide.‡ was given 10 mg/kg subcutaneously at the same time as egg white.

SY-28 in the dose employed, protects normal and adrenalectomized rats against edema and death, although it was by itself toxic to adrenalectomized rats. "SY-28" is a potent antihistaminic[13,15] in addition to being a very effective adrenergic blocking drug,[13,18] as are several other β-haloalkylamines.[13-15,17,19] Since it is known that antihistaminics inhibit egg white edema in rats with intact adrenals,[1,10,11] it was felt that experiments

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‡ Generously supplied by Dr. G. Rieveschl, Jr., Parke, Davis and Co., Detroit, Mich.

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TABLE V. Exp. 5.

Group	% incidence of edema	Degree of edema	% dead	Symptoms
		1 cc egg white		
Adx saline controls	100	2.0	100	Severe cyanosis
” phenergan, 5 mg/kg	60	1.5	20	Cyanosis
” ” 10 mg/kg	100	2.0	20	Some cyanosis
” ” 25 mg/kg	60	1.0	0	None
		No egg white		
Adx saline controls	—	—	0	”
” phenergan, 5 mg/kg	—	—	0	”
” ” 10 mg/kg	—	—	0	”
” ” 25 mg/kg	—	—	0	”

should be performed in which an antihistamic as well as a purely adrenergic blocking agent was used in adrenal insufficient animals. As an antihistaminic, Phenergan[§] (N-dimethylamino-2-propyl-1-thiodiphenylamine, "32 77-RP") was selected because of its potent antihistaminic action and its anti-capillary permeability effects,[12,20-30] and because in the previous report[1] it was found to be a highly effective inhibitor of egg white edema in the normal rat.

Exp. 5. Eight groups of 5 males each were adrenalectomized 8-10 days prior to the experiment and maintained on salt until 24 hr prior to the experiment, at which time water was substituted. The average body weight at the time of the experiment was 175 g. Phenergan in 2 cc volume was given subcutaneously to the first 4 groups in doses of 5, 10 and 25 mg/kg simultaneously with 1 cc egg white intraperitoneally, and in similar doses to the last 4 groups without egg white, in order to see if the doses of drug given in the absence of egg white were toxic in adrenal insufficient rats. The symptoms were graded 2 hours later.

Phenergan, a potent anthistaminic and anticapillary "permeability" drug, effectively antagonizes egg white toxicity in adrenal in-

TABLE VI. Exp. 6.

Group (normal rats)	% incidence of edema	Degree of edema
1. Egg white, 1 cc /kg	0	0
2. ” ” 2 ”	0	0
3. ” ” 3 ”	0	0
4. ” ” 4 ”	30	2.0+

[§] Generously supplied by Drs. A. Gibson and R. C. Pogge of Merck and Co., Inc., Rahway, N. J.

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[‖] In order to rule out the possibility that Phenergan protected *normal* rats against egg white edema, as previously reported,[1] via epinephrine release from the adrenal medulla, experiments were performed on 10 normal and 10 adrenal demedullated rats given 2 cc/kg egg white intraperitoneally, and with and without 2.5 mg/kg Phenergan subcutaneously 1 hour prior to the egg white. There were no differences in the incidence, onset and intensity of the resulting severe edema, in confirmation of our previous work in which it was found that 10 mg/kg was inhibitory.[1] In another experiment on 20 adrenal demedullated rats, however, 25 mg/kg of Phenergan completely inhibited the edema, whereas the untreated animals had marked edema. This experiment therefore rules out the possibility, suggested by the nearly convulsive doses of antihistaminics used by Leger and Masson,[10] and Brown and Werner,[11] that the antihistaminics act by releasing epinephrine from the adrenal glands.

TABLE VII. Exp. 7.

Group	Egg white, cc/kg	% incidence of edema	Degree of edema	% dead
1. Normal controls	2	100	3+	0
2. ” ”	4	100	3	0
3. Normal, + SKF-501	2	83	2	0
4. ” ”	4	83	2	0
5. Adx controls	2	83	2	33
6. ” ”	4	83	2	83
7. ” + SKF-501	2	100	2	17
8. ” + ”	4	83	2	33

sufficient rats‖ and does so in doses which are not toxic in adrenalectomized rats not given egg white. As an adrenergic blocking agent without antihistaminic action, “SKF-501,”¶ N-(9-fluorenyl)-N-ethyl-β-chloroethylamine, was chosen since it is an even more powerful yet less toxic adrenergic blocking drug than Dibenamine itself,[31] but has little or no antihistaminic activity,** as might be predicted on the basis that in over 100 compounds tested by Nickerson,[16–18] only α-naphthylmethyl- and β-phenoxyethyl derivatives of the β-haloakylamines were found to be effective antihistaminics.

Exp. 6. Four groups of 3 normal male rats each, average body weight 310 g were given egg white, 1, 2, 3 and 4 cc/kg, respectively, of 1:1 egg white-saline mixture intraperitoneally 3 hours after 2 mg/kg SKF-501 intravenously. The symptoms were graded 2 and 3 hours later.

SKF-501, a purely adrenergic blocking agent, inhibits egg white edema in normal rats.

Exp. 7. Forty-eight male rats were divided into 8 groups of 6 each, with an average body weight of 310 g. The operated groups were adrenalectomized 7 days prior to the experiment and were maintained on salt until 24 hours prior to the experiment, at which time water was substituted. The drug-treated groups were given 2 mg/kg SKF-501 intravenously 1 hour before egg white. The egg white was given in 2 doses, 2 cc/kg and 4 cc/kg intraperitoneally, and the symptoms were graded 3 hours later.

SKF-501, a purely adrenergic blocking drug, decreases the mortality caused by egg white edema in adrenal insufficient rats. The protection in normal controls is somewhat less when given 1 hour before than when given 3 hours before as in Exp. 6.

Discussion. The edema and toxicity induced in normal and adrenal insufficient rats by the intraperitoneal injection of egg white is inhibited by epinephrine and arterenol and probably other pressor substances, but not by Isuprel,†† 1-(3′,4′-dihydroxyphenyl) - 2 - isopropylaminoethanol-HCl,[1] (*present paper*). The cephalic edema caused by *p*-phenylenediamine is inhibited by epinephrine, tyramine, phenylethanolamine, drugs which release endogenous epinephrine such as strychnine and nicotine, and by sympathetic stimulation.[32–36] Epinephrine also relieves the edema associated with urticaria and angioneurotic edema. In adrenal insufficiency the increased capillary filtration of fluids induced by traumatic shock, intraperitoneal administration of hypertonic solutions, egg white, and in all probability *p*-phenylenediamine, intravenous hyaluronidase[37] and other conditions, probably all lead to hypotensive shock as a result of an uncompensated decreased blood volume. Epinephrine may antagonize all of those effects by its arteriolar vasoconstrictive action; and in addition protects adrenalectomized animals against histamine and anaphylactic shock. Antihistaminics inhibit egg white edema, hyaluronidase edema,[38] urticarias, angioneurotic edema and histamine and anaphylactic shock probably both by antagonizing the action of released histamine and by decreasing capillary filtration by arteriolar vasoconstriction. No explanation can be given for the protective effect of adrenergic blockade in egg white edema. At first thought, it might have been anticipated that it would aggravate the condition by blocking the protective action of endogenous epinephrine and sympathin-N. This is not the case, however, and the fact the adrenal demedullation also is not detrimental is confirmatory of this lack of effect. It is possible that SFK-501 and the adrenolytic component of SY-28 protect in the same way that Dibenamine does in hemorrhagic and traumatic shock, as demonstrated by Remington,[39,40] although the mode of action in both cases is unknown. The effect suggests, however, the participation of an adrenergic component in egg white edema, as seems to be the case in the pulmonary edemas induced by ammonium chloride[41,42] and increased intracranial pressure or brain concussion.[43]

¶ Generously supplied by Dr. Glen E. Ullyot of Smith, Kline and French Laboratories, Philadelphia, Pa.

** As tested by the prevention of histamine-induced spasm of the isolated guinea pig intestine.[16]

†† Not investigated in adrenalectomized rats.

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[33] Tainter, M. L., and Hanzlik, P. J., *J. Pharm. Exp. Therap.*, 1924, **24**, 179.

[34] Gibbs, O. S., *J. Pharm. Exp. Therap.*, 1923, **20**, 221; 1931, **42**, 65.

[35] Tainter, M. L., *J. Pharm. Exp. Therap.*, 1926, **27**, 201; 1928, **33**, 129.

[36] Cohen, M. B., Wasserman, P., and Rudolph, J. A., *J. Pharm. Exp. Therap.*, 1933, **48**, 235.

[37] Elster, S. K., Freeman, M. E., and Dorfman, A., *Am. J. Physiol.*, 1949, **156**, 429.

Summary. Epinephrine protects adrenal insufficient rats from the edema and mortality caused by the intraperitoneal administration of egg white. Mortality is prevented by doses (0.04 mg/kg) which are not able to prevent edema in rats with intact adrenals.

The antihistaminic Phenergan, N-dimethylamino-2-propyl-1-thiodiphenylamine ("3277 RP"), in doses as low as 5 mg/kg prevent death from egg white edema in adrenalectomized rats.

"SY-28", N-(2-bromoethyl)-1-naphthalenemethylamine hydrobromide, a Dibenamine congener possessing both antihistaminic and adrenergic blocking activity, also prevents the edema and death, but is in itself toxic to adrenalectomized rats.

"SKF-501", N-(9-fluorenyl)-N-ethyl-β-chloroethylamine, a Dibenamine cogener with potent adrenergic blocking powers but with a low toxicity and without antihistamine action, also inhibits the edema and decreases the mortality.

The possible modes of action are discussed.

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[38] Elster, S. K., Freeman, M. E., and Lowry, E. L., *J. Pharm. Exp. Therap.*, 1949, **96**, 332.

[39] Remington, J. W., Wheeler, N. C., Boyd, G. H., Jr., and Caddell, H. M., Proc. Soc. Exp. Biol. and Med., 1948, **69**, 146.

[40] Remington, J. W., *Fed. Proc.*, 1949, **8**, 131.

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Received October 18, 1949. P.S.E.B.M., 1949, **72**.

A Microbiological Assay for Vitamin B_{12} Using *Lactobacillus leichmannii*.*

(17485)

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Lactobacillus leichmannii (ATCC 4797) has been reported by several laboratories[1–3] to be a useful test organism for the microbiological measurement of vitamin B_{12}. Skeggs, *et al.*[1] found ascorbic acid and air to stimulate a growth response in this organism in the absence of vitamin B_{12} on a basal medium containing casein hydrolysates, but were able to minimize these effects by autoclaving the test assay for 15 minutes at 120°C. Other workers[3] also found certain reducing agents to promote a growth response in both *L. leichmannii* (ATCC 4797) and *L. leichmannii* (ATCC 7830) in the absence of vitamin B_{12} on casein hydrolysate media, but in addition found that these agents were able to increase the growth response produced by vitamin B_{12}. In attempting to overcome these difficulties, a microbiological assay has been developed which is highly sensitive to vitamin B_{12} and which yields consistent results. The experimental basis and the details of the procedure are presented in this report.

Assay Procedure. The culture of *Lactobacillus leichmannii* 4797 used in this assay was obtained from the American Type Culture Collection, Georgetown University, Washington, D. C. *L. leichmannii* (ATCC 7830) has also been used successfully in this assay. The culture is maintained in a stab medium composed of 5 ml of double strength basal medium (Table I), 0.3 unit of 15 unit USP injectable liver extract, 150 mg of agar and distilled water to make 10 ml. Each week it is transferred to a broth medium[4] in which it is grown for 24 hours at 37°C and is then taken back to the stab medium. The inoculum is prepared by growing the organism for 24 hours in a broth medium having the same composition as the stab medium except for the omission of agar. The procedure for the inoculation and incubation of the assay is the same as that described by Daniel, *et al.*[5] except that the diluted inoculum is made to read 60 on the galvanometer scale of a Coleman spectrophotometer at wave length 650 mμ. The composition of the amino acid medium used in this method is presented in Table I. It is a modification of the medium used by Daniel, *et al.*[5] in studies on the nutrition of *L. casei* and that of Snell, *et al.*[6] for *L. leichmannii.*

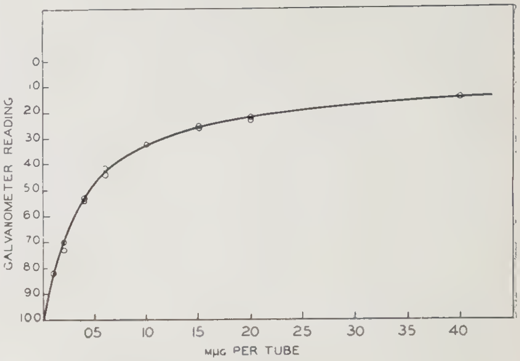

FIG. 1.
Response of *Lactobacillus leichmannii* to crystalline vit. B_{12}.

After all constituents of the medium are combined, the pH is adjusted to 5.5 with 20% potassium hydroxide. The volume is then made to 1 liter and the medium steamed for 5 minutes. Five ml of the double-strength basal medium and the sample to be assayed are placed in the assay tubes. The final volume is made to 10 ml. The assay is incubated at 37°C for 16 hours or until the tube containing 0.1 mγ of vitamin B_{12} reaches a turbidimetric reading of approximately 30 on the galvanometer scale of the spectrophotometer. At this time the turbidity of the cell suspension is read by placing the inoculated blanks at 100 on the galvanometer scale. Very little growth, if any, is ordinarily present in the blanks. A typical standard curve is shown in Fig. 1. The results show that on this basal medium the assay range is between 0.001 and 0.04 mγ of vitamin B_{12} per ml of medium.

The canned tomato juice used in this method is purchased on the open market. It is filtered through No. 617 Eaton and Dikeman[†]

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[*] This work was undertaken in cooperation with the Office of Naval Research, Navy Department, Washington, D.C., and was aided by grants to Cornell University by the Nutrition Foundation, Inc., New York, the Cerophyl Laboratories, Inc., Kansas City, Mo., The Commercial Solvents Corporation, New York City, The Cooperative G.L.F. Exchange, Ithaca, N. Y., and the Western Condensing Company, San Francisco, Calif. This work was conducted in the Nutrition Laboratories of the Department of Poultry Husbandry. The technical assistance of Betty F. Brown and Diana M. Cameron is acknowledged.

[1] Skeggs, H. R., Huff, J. W., Wright, L. D., and Bosshardt, D. K., *J. Biol. Chem.*, 1948, **176**, 1459.

[2] Capps, B. F., Hobbs, N. L., and Fox, S. H., *J. Biol. Chem.*, 1949, **178**, 517.

[3] Stokstad, E. L. R., Dornbush, A. C., Franklin, A. L., Hoffman, C. E., Hutchings, B. L., and Jukes, T. H., *Fed. Proc.*, 1949, **8**, 257.

[4] Nymon, M. C., Gunsalus, I. C., and Gortner, W. A., *Science*, 1945, **102**, 125.

[5] Daniel, L. J., Scott, M. L., Heuser, G. F., and Norris, L. C., *J. Biol. Chem.*, 1948, **174**, 71.

[6] Snell, E. E., Kitay, E., and McNutt, W. S., *J. Biol. Chem.*, 1948, **175**, 473.

filter paper on a Büchner funnel with the aid of Celite filter aid (Johns-Manville). The resulting solution is adsorbed with 12.5 g of activated norit per liter at the natural pH of the serum (4.0 to 4.2) for ½ hour and filtered to remove the norit.

Development of Assay. Crystalline vitamin B_{12}‡ and a potent vitamin B_{12} concentrate[7] were used in the development of this assay. The vitamin B_{12} concentrate was prepared from 15 unit USP injectable liver extract by the technic of counter-current distribution as described by Craig.[8] The results of a study showing the need of *L. leichmannii* for a growth factor present in tomato juice is presented in Table II. Crystalline vitamin B_{12} was observed to produce a submaximal plateau response when assayed on the amino acid medium containing no tomato juice. The addition of adsorbed tomato juice filtrate promoted a much improved growth response from the vitamin especially at the higher levels. Increasing the content of ferrous sulfate and cysteine, or adding ascorbic acid or sodium thioglycollate to the medium, did not replace the need for the supplement contrary to the findings of Stokstad, *et al.*[3] The same results were obtained when lyophilized tomato juice filtrate was employed.

Preliminary experiments showed also that a phenol-n-butyl alcohol extract of USP injectable liver extract which had been freed of vitamin B_{12} and thymidine is capable of replacing tomato juice as a source of unknown factors in the assay medium. Other studies indicated that the factor for *L. leichmannii* and that reported by Daniel, *et al.*[5,9] for *L. casei* are concentrated by counter-current distribution in the same fractions of injectable liver extracts and therefore may be identical. Whether this factor is the same as that reported by Shorb[10] has not been determined.

TABLE I.
Double-Strength Medium* for Assay of Vit. B_{12} Using *Lactobacillus leichmannii.*

DL-Alanine	425	mg
L-Arginine	425	"
DL-Aspartic acid	425	"
L-Cystine	400	"
L-Glutamic acid	1213	"
Glycine	425	"
L-Histidine	425	"
L-Hydroxyproline	50	"
DL-Isoleucine	425	"
DL-Leucine	425	"
L-Lysine	213	"
DL-Methionine	425	"
DL-Norleucine	425	"
DL-Phenylalanine	100	"
L-Proline	425	"
DL-Serine	50	"
DL-Threonine	50	"
DL-Tryptophan	50	"
L-Tyrosine	400	"
DL-Valine	425	"
Asparagine	150	"
Glutamine	200	"
Glucose	40	g
Cysteine hydrochloride	2	"
Sodium acetate (trihydrate)	12	"
Sodium citrate	10	"
$MgSO_4 \cdot 7H_2O$	2.8	"
$FeSO_4 \cdot 7H_2O$	800	mg
$MnSO_4 \cdot 4H_2O$	600	"
KH_2PO_4	4	g
K_2HPO_4	4	"
Adenine sulfate	10	mg
Guanine hydrochloride	10	"
Uracil	10	"
Xanthine	15	"
Tween 80†	2	ml
Tomato juice filtrate‡	600	"
Pyridoxine	4	mg
Pyridoxal	4	"
Niacin	2	"
Riboflavin	2	"
Thiamine	2	"
Calcium pantothenate	2	"
Biotin	10	γ
Folic acid	110	"
Pyridoxamine	800	"
p-Aminobenzoic acid	80	"
Distilled water to make	1000	ml

* After preparation the pH of the medium is adjusted to 5.5 with 20% potassium hydroxide, steamed 5 minutes and cooled.

† Polyoxyethylene sorbitan monooleate (Atlas Powder Co.).

‡ Prepared as described previously.

During some early studies it was found that better growth is obtained with *L. leichmannii* at the same level of vitamin B_{12} when the initial pH of the medium was more acidic than the usual value of 6.6 to 6.8.[1,2,6] In

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† The Eaton-Dikeman Company, Mt. Holly Springs, Pa.

‡ The crystalline vitamin B_{12} used in this work was kindly supplied by Dr. E. Lester Smith of Glaxo Laboratories, England, and Dr. D. F. Green of Merck and Co., Rahway, N. J.

[7] Carlson, C. W., Thesis, Cornell University.

[8] Craig, L. C., *J. Biol. Chem.*, 1944, **155**, 519.

[9] Daniel, L. J., Peeler, H. T., Norris, L. C., and Scott, M. L., *J. Biol. Chem.*, 1949, **177**, 917.

[10] Shorb, Mary S., *J. Bact.*, 1947, **53**, 669.

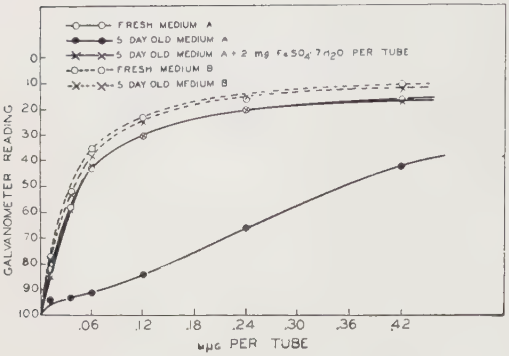

FIG. 2.

Response of *Lactobacillus leichmannii* to vit. B_{12} concentrate on fresh and aged media. Medium A contains no cysteine and only 1.11 mg per tube of $FeSO_4 \cdot 7H_2O$. Medium B contains 4 mg per tube of $FeSO_4 \cdot 7H_2O$ and 10 mg per tube of cysteine.

view of this finding, studies were undertaken to establish the optimum pH range for this organism on the amino acid medium. Nine samples of the medium were used. Each contained vitamin B_{12} in such quantity that, on dispensing, every assay tube had 0.4 mγ of vitamin B_{12} . The pH of these samples was adjusted so as to have a graded series ranging from 4.0 to 7.0. The results showed that under these conditions maximum growth was progressively retarded at pH values greater, or less, than pH 5.5.

On a medium prepared as shown in Table I, but containing no cysteine and only 222 mg of $FeSO_4 \cdot 7H_2O$ per liter, poorer growth was obtained at the same level of vitamin B_{12} when the medium was a few days old than when it was prepared fresh. This effect became marked if a vitamin B_{12} concentrate prepared from a 15 unit USP injectable liver extract was used as the supplement. In attempting to overcome the effect of aging, the addition of 2 mg of ferrous sulphate per tube to the aged medium was found to restore it to a "fresh" condition. Response curves demonstrating this are shown in Fig. 2.

Studies were then undertaken to determine how to preserve the medium in a fresh condition for longer periods of time. In these experiments 0.118 mγ of vitamin B_{12} concentrate per tube was assayed in the presence of graded levels of $FeSO_4 \cdot 7H_2O$ and cysteine. The results of the study showed that the growth response reached a plateau with either 4 mg of ferrous sulfate, or 15 mg of cysteine per tube. A combination of 4 mg of ferrous sulfate per tube and graded levels of cysteine, however, promoted an additional growth response which reached a plateau with only 4 mg of cysteine per tube. Additional cysteine had no effect on growth but aided in maintaining the medium in a fresh state for a longer period of time. As a consequence of these findings, the composition of the medium was changed so that it contains 4 mg of $FeSO_4 \cdot 7H_2O$ and 10 mg of cysteine per tube. The effect of allowing the medium containing the increased iron and cysteine to age 5 days before use is also shown in Fig. 2.

Certain discrepancies were found to exist between the assays conducted on the amino acid medium described in this report and those conducted on a medium prepared as described by Skeggs, *et al.*[1] The responses of crystalline vitamin B_{12} and a vitamin B_{12} concentrate using these media are presented in Table III. The values from the amino acid medium were obtained at 16 hours and those on the casein hydrolysate medium at 24 hours.[1] In each case more growth resulted from the same level of vitamin B_{12} on the amino acid medium than on the other. Furthermore, the vitamin B_{12} concentrate which was shown to be highly active for the chick[11] promoted only slight response on the casein hydrolysate basal medium. On investigating the cause of this discrepancy it was found that addition of ferrous sulfate to the casein hydrolysate medium markedly increased the growth response from the vitamin B_{12} concentrate which is in agreement with the work of Stokstad, *et al.*[3] The assay blanks were found to have considerable growth under these conditions. This growth was found to be due to the hydrolyzed casein in the medium and indicated that casein hydrolysate contained a form of vitamin B_{12} active only in the presence of reducing agents. This is probably the same effect noted by Welch, *et al.*[12]

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[11] Carlson, C. W., Miller, R. F., Peeler, H. T., Norris, L. C., and Heuser, G. F., *Poultry Sci.*, 1949, **28**, 750.

[12] Welch, A. D., and Wilson, F. F., *Arch. Biochem.*, 1949, **22**, 486.

TABLE II.
Response of *L. leichmannii* to Vit. B_{12} in Presence and Absence of an Unidentified Growth Factor in the Medium.

Substance	Level per tube mγ	Galvanometer readings* Medium without tomato juice filtrate	 Medium with tomato juice filtrate
None		100	100
Crystalline vit. B_{12}	.012	85	80
	.036	75	57
	.059	66	42
	.118	57	26
	.236	50	20
	.412	53	13

* A galvanometer reading of 100 represents no growth.

TABLE III.
Comparison of Two Different Media in the Assay of Vit. B_{12}.

Substance	Level per tube, mγ	Galvanometer readings* Amino acid medium†	 Casein-hydrolysate medium‡
None		100	100
Crystalline vit. B_{12}	.012	76	95
	.036	49	88
	.059	35	79
	.118	26	61
	.236	16	40
	.412	13	24
Vit. B_{12} conc.§	.012	74	98
	.036	50	93
	.059	36	90
	.118	23	93
	.236	15	83
	.412	11	74
Adsorbed enzyme digested casein‖	mg 25	18	—

* A galvanometer reading of 100 means no growth.
† The amino acid medium reported in this paper.
‡ Prepared as described by Skeggs *et al., J. Biol. Chem.*, 1948, **176**, 1459.
§ Prepared as described previously.
‖ A constituent of the casein-hydrolysate medium. Adsorbed three times the amount reported necessary by Skeggs *et al., J. Biol. Chem.*, 1948, **176**, 1459.

The results of studies on the recovery of vitamin B_{12} from USP injectable liver extract are presented in Table IV. The results showed a mean recovery of 100.8% from this product. These results were confirmed by conducting recoveries from Wilson's liver fraction L. In this case individual recoveries ranged from 96.7 to 109.4% with a mean recovery of 100.7%. No synergism was found to exist between the natural materials and crystalline vitamin B_{12}. The assay method, therefore, appears to be specific for vitamin B_{12} activity. Further evidence of this was obtained in counter-current distribution studies of injectable liver extract since the assay of the fractions in the various tubes indicated the partition of a single substance.

Thymidine,§ which was shown to promote

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§ The thymidine used in these studies was kindly supplied by Dr. William Shive of the University of Texas.

TABLE IV.
Recoveries of Crystalline Vit. B_{12} Added to Liver Fractions.

Substance	Level per tube, units	Vit. B_{12} by assay, mγ	Added B_{12} per tube, mγ	Theoretical B_{12} per tube, mγ	Actual B_{12} per tube, mγ	Recovery, %	Standard error of the mean
USP injectable liver extr. (15 unit)	.000012	.008	.012	.020	.021	105.0	
	.000025	.012	.012	.024	.026	108.3	
	.00005	.025	.012	.037	.035	94.6	
	.000075	.037	.012	.049	.051	104.1	
	.0001	.051	.012	.063	.058	92.1	
						100.8	± 3.154

TABLE V.
Uniformity of Vit. B_{12} Assay Values Obtained from Various Substances with *L. leichmannii.*

Substance	No. of assays	Vit. B_{12} content	Range	Standard error of the mean	Coefficient of variation, %
Vit. B_{12} conc.*	14	8.06 γ/ml	7.69 –8.54	± 0.067	3.10
USP injectable liver extr. (15 unit)	10	0.590 γ/unit	0.545–0.629	± 0.009	4.64
Wilson's liver L	7	0.389 γ/g	0.374–0.416	± 0.006	3.62

* Prepared as described previously.

a growth response in *L. leichmannii* on a casein hydrolysate medium,[1] was also found to promote a growth response on the amino acid medium. Twenty-four thousand times more thymidine than vitamin B_{12} was required to produce the same growth response. Due to this wide ratio, it is highly improbable that interference from thymidine will be encountered in the assay of most materials for vitamin B_{12} activity by the procedure described in this report.

The ability of the method to produce repeatable assay results was determined by running several assays on successive days on the same sample of unknown against crystalline vitamin B_{12} solution. The results are presented in Table V. Excellent replicability was found to exist between repeated daily assays. Values from a crude liver fraction were just as consistent as those from a vitamin B_{12} concentrate.

Assay of Various Materials. Feedstuffs currently employed in experimental chick diets used to study vitamin B_{12} at this laboratory were chosen for the development of extraction procedures. The vitamin B_{12} content of several of these materials is presented in Table VI.

USP injectable liver extracts were found to need no treatment other than dilution. A range of 87.1 to 2170 mγ of vitamin B_{12} per USP unit was found from the assay of 10 different products. The results confirm the report by Rickes, *et al.*[13] that a considerable variation exists in the amount of vitamin B_{12} to which 1 USP unit is equivalent. In the liberation of vitamin B_{12} from animal products 1 g sample is placed in a 125 ml Erlenmeyer flask together with 50 ml of phosphate buffer (pH 6.8), 20 mg of pancreatin suspended in phosphate buffer, and 1 ml of toluene. The materials are well mixed and incubated for 20 hours at 37°C. After incubation the pH is adjusted to 5.5 with 2 N HCl followed by 5 minutes of steaming. The sample is cooled, adjusted to 100 ml and filtered through Whatman fluted filter paper No. 12. With vegetable materials a 2 g sample is placed in a 250 ml Erlenmeyer flask together with 100 ml of acetate buffer (pH 4.5). The mixture is autoclaved for 30 minutes at 20 lb. After cooling, a suspension containing 40 mg of papain and 40 mg of

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[13] Rickes, E. L., Brink, N. G., Koniuszy, F. R., Wood, T. R., and Folkers, K., *Science*, 1948, **107**, 396.

TABLE VI.
Vit. B_{12} Content of Several Materials.

Material	Vit. B_{12} content, $m\gamma/g$
Soybean meal	11
Yellow corn	10
Wheat	7
Alfalfa leaf meal	40
Dried brewers' yeast	8
Wilson's liver L	389
Crude casein	104
White fish meal	98.3
Red fish meal	111
Condensed fish solubles	92
Meat scraps	42.3

takadiastase is added to the mixture together with 2 mg of neutralized cysteine hydrochloride and 1 ml of toluene. The materials are well mixed and incubated for 40 hours at 37°C. After incubation the samples are adjusted to pH 5.5, steamed 5 minutes, cooled, adjusted to 200 ml and filtered through Whatman fluted filter paper No. 12.

Other treatments used in an attempt to liberate the vitamin include autoclaving at acidic and neutral pH, allowing the samples to autolyze for 20 hours in acetate buffer, steaming 30 minutes in acetate buffer and allowing the sample to stand in distilled water. These treatments did not liberate as much vitamin B_{12} as was obtained by the methods outlined in detail. Treatment of the animal products with trypsin or pepsin produced the same results as were obtained with pancreatin. However, pancreatin is used in the procedure because less growth occurred in the enzyme assay blanks.

Summary. A microbiological assay for vitamin B_{12} using *Lactobacillus leichmannii* (ATCC 4797) has been developed. The assay medium contains crystalline amino acids as the nitrogen source and adsorbed tomato juice filtrate as a source of unidentified growth factors. A high level of cysteine and ferrous sulfate are present in the medium to keep it in the necessary reduced state. Data are given to show the degree of precision and reproducibility to be expected from the assay.

Received Sept. 3, 1949. P.S.E.B.M., 1949, **72.**